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13. ABSTRACT ***(Maximum 200 Words)***

Botulinum neurotoxin (BoNT) types A and B selectively block transmitter release by cleavage of SNAP-25 and synaptobrevin, respectively; many months are required for full recovery from the resultant neuromuscular paralysis. To decipher the molecular basis for such prolonged poisoning, intoxication of adreno-chromaffin cells was monitored over 2 months. Exocytosis in BoNT/B-treated cells resumed after 56 days due to the appearance of intact synaptobrevin. However, inhibition continued in BoNT/A-treated cells, over the same interval, due to the persistence of cleaved SNAP-25 (1-197). When recovery of exocytosis was attempted by transfection of poisoned cells with the gene encoding full-length SNAP-25 (1-206), no restoration of exocytosis ensued even 3 weeks post intoxication. To ascertain if this failure was due to the persistence of the toxin's protease activity, the cells were transfected with genes encoding mutated forms of SNAP-25, engineered (via point-mutations at residues Q197 and/or R198) to be highly resistant to BoNT/A protease. Importantly, expression of these mutants yielded complete rescue of exocytosis, even 3 weeks after the initial exposure to BoNT/A. Thus, this unusually long-term persistence of protease activity is a major contributing factor to the extended duration of BoNT/A poisoning. These novel findings establish the proof of principle for fast and complete rescue from BoNT/A intoxication by an innovative and straightforward transfection process. Moreover, such a fundamental advance provides the realistic potential of a new and effective therapy for botulism, particularly, because the technology for gene targeting is available.

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Professor J. Oliver Dolby 13th Oct 99

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INTRODUCTION

As a prelude to our prime goal of developing an effective therapy for poisoning with botulinum neurotoxin type A (BoNT/A), the contributions of amino acids at the P1, P′1 or P′2 positions in SNAP-25 to its interaction with the active site in the light chain (LC) of BoNT/A have been assessed by site-directed mutagenesis. Wild-type and mutated SNAP-25 were expressed in *E. coli* as glutathionine-S-transferase (GST)-linked proteins as detailed in the 1st annual report. Herein, the proteolytic susceptibilities of the resultant mutated variants of SNAP-25 to BoNT/A were determined using the ELISA methods, also described earlier. Whilst some of these mutants are similar to those prepared previously by Schmidt & Bostian (1997) [where changes were made to a C-terminal 17-mer (187-203) of SNAP-25], attention was focussed here on the full-length substrate because of the expectation of this being cleaved more efficiently; additional mutations were also investigated. In fact, this study demonstrated a much faster BoNT/A cleavage rates for the full-sized substrate compared to smaller synthetic peptides that encompass the cleavage site but lack an important upstream S4-motif (Rossetto *et al.*, 1994; Washbourne *et al.*, 1997). The overall aim was to produce SNAP-25 gene constructs encoding variants highly-resistant to proteolysis by BoNT/A which might retain ability to support synaptic vesicle docking and fusion when expressed in neurons; these could then be used to examine the molecular basis for the blockade of exocytosis persisting for so many weeks. Ideally, SNAP-25 proteins with single-, double- or triple-point mutations at positions P2 through to P′2 could rescue neurotransmitter release in BoNT/A-paralysed nerves if successful methods of targeted-delivery were developed.

Whilst the mechanism of BoNT-induced inhibition has received a lot of attention, the molecular processes involved in recovery from poisoning, which can take several months depending on which serotype is used, remains poorly understood. In humans, the improvement from neuromuscular paralysis, 49 days post-injection with either BoNT/A- or B is ~ 10% and ~70%, respectively (Sloop *et al.*, 1997). Recently, the physiological basis for this eventual resumption of neurotransmission in poisoned motor-end-plates has been revealed by elegant *in vivo* monitoring of synaptic function in the same identified poisoned nerve endings of living mice (de Paiva *et al.*, 1999). These studies proved that full recovery relies on return of synaptic function to the originally-poisoned nerve terminals, though extra-junctional synapses created by newly-formed nerve sprouts underlie the first phase of recovery of neuromuscular transmission. However, it is still unclear if and how the turnover of SNAP-25 and the BoNT/A-truncated form affect the recovery process.

In order to understand the molecular basis of this protracted intoxication, which seems to vary with the serotype used, a similar study was undertaken in isolated adreno-chromaffin cells. Evoked exocytosis from this convenient cell model shares many characteristics with synaptic vesicle-mediated neurotransmitter release in neurons; in particular, SNAP-25, synaptobrevin (Sbr), and syntaxin1 are present and have all been shown to be essential for large dense-core vesicle (LDCV) exocytosis (for review see Morgan and Burgoyne, 1997). Further, BoNT/A- and /B- induced blockade of exocytosis in chromaffin cells has been shown to be due to cleavage of SNAP-25 and Sbr/Cbr, respectively (Lawrence *et al.*, 1996). Finally, previous work on this system had suggested that inhibition of catecholamine release by BoNT/A or TeTx persists for prolonged periods (Bartels *et al.*, 1994). Despite these encouraging features, the validity of using chromaffin cells for studying recovery of release had to be assessed in relation to data reported for the neuromuscular junction (NMJ). In particular, the different profiles of recovery for BoNT/A and B found in humans (Sloop *et al.*, 1997) needed to be determined in chromaffin cells. This would allow the turnover of the respective BoNT-truncated SNAREs to be directly assessed, a question that cannot be easily answered at the motor nerve terminals. Further, it was hoped to rescue release in BoNT/A-poisoned chromaffin cells through the introduction of vectors expressing wild-type SNAP-25. The failure to achieve this feat provided not only proof for the continued activity of the toxin but also implied that the SNAP-25 needed to be mutated in such a way that it was profoundly resistant to BoNT/A without affecting its ability to support exocytosis. The successful production of BoNT/A-resistant SNAP-25s that are capable of rescuing release establishes that normal release can be restored at any point after BoNT/A poisoning and that these residues are redundant for exocytosis.

BODY

MATERIALS AND METHODS

Materials. Highly purified BoNT/A, /B and /E were supplied by Drs Gary Lawrence (Imperial College, London) and Bibhuti DasGupta (Madison, Wisconsin). An affinity-purified polyclonal antibody specifically recognising the N-terminal product resulting from BoNT/A-mediated cleavage of SNAP-25 (termed anti-SNAP-25A-pAb) was prepared as detailed in the 1st annual report. Antibody preparations reactive with C-terminus of SNAP-25 were raised against a synthetic peptide encompassing residues 195-206 and affinity-purified on the immobilised immunogen. Urografin (Schering Healthcare, G.D.R.), digitonin (Novabiochem, U.K.), radio-immunoassay (RIA) kit for human growth hormone (hGH) (Nichols Institute, San Capistrano, CA, USA), Quick-Change™ (Stratagene, Netherlands),

calcium phosphate reagents (GIBCO-BRL, Scotland, U.K.), Superfect™ (Qiagen, U.K.), pGEX-2T (Amersham Pharmacia, U.K.) and pcDNA1.1 (Invitrogen, The Netherlands) were purchased.

Culture of cells and intoxication with BoNT/A or /B. Bovine chromaffin cells were prepared from adrenal glands and maintained as primary cultures as described previously (Lawrence *et al.*, 1996). Cells required for transfection were further enriched (to remove any contaminating fibroblasts) using Urografin density-gradient centrifugation, as detailed by Wilson (1987). Within 2-3 days after preparation, cells were incubated with low ionic strength medium (LISM) at 37°C for 24 h in the absence or presence of 6.6 nM BoNT/A or 66 nM BoNT/B and maintained at 37°C for up to 56 days by weekly replacement of medium (Lawrence *et al.*, 1996).

Stimulation and quantification of catecholamine secretion from intact chromaffin cells. Immediately before measuring release, cells pre-treated with or without BoNT/A or /B were briefly washed with Locke's solution (Wilson, 1987) and then incubated in quadruplicate at 22°C in this buffer with and without the inclusion of 2 mM Ba^{2+}. After 15 min, aliquots of the medium bathing the cells were removed and assayed for catecholamine content, using a fluorometric procedure (Lawrence *et al.*, 1996). Values for basal release of catecholamine were subtracted from the respective evoked component and expressed as a percentage of the total content.

Expression of wild-type or mutant SNAP-25 in cells : sole monitoring of transfected cells using a hGH-based reporter system. Wild-type or mutated SNAP-25 genes generated by PCR-based mutagenesis techniques (see first annual report; listed in Figure 1) were introduced into the mammalian expression vector pcDNA1.1/Amp from pGEX-2T, using the *Bam*H1 and *Eco*R1 restriction sites. Control or 6.6 nM BoNT/A-treated chromaffin cells were transfected with the hGH construct together with that of a control, chloramphenicol acetyl transferase (pcDNA1.1/Amp-CAT) or the appropriate SNAP-25 gene, using the calcium phosphate precipitation method (Holz *et al.*, 1995). Four to six days after transfection, hGH secretion from intact chromaffin cells was stimulated and quantified as described for catecholamine release except for the use of a RIA. Some transfected cells were permeabilised with 20 μM digitonin in a permeabilisation buffer and exposed for 15 min to reduced BoNT/E. Evoked release was assessed over a subsequent 15 min by the

addition of 20 μM free Ca^{2+} (Lawrence *et al.*, 1997). Aliquots were removed and assayed for hGH content; Ca^{2+}-evoked hGH secretion was calculated as described above.

Assessment of the proteolytic activities of BoNT/A or /B in cell cultures. CHO cells, which lack SNAP-25, were plated at 50-70% confluency on a 6 well plate. After treatment with BoNT/A in LISM as detailed for chromaffin cells, CHO cells were transfected with pcDNA1.1/Amp-SNAP-25s using Superfect™. Membranes were isolated from the latter, and chromaffin cell samples, as previously described (Lawrence *et al.*, 1996; Foran *et al.*, 1996), subjected to immunoblotting with IgGs including anti-SNAP-25 (recombinant)-IgG (an antibody raised against full-length recombinant GST-SNAP-25), anti-$SNAP\text{-}25_A$-pAb (an antiserum solely reactive with $SNAP\text{-}25_A$), anti-SNAP-25 (C-terminus)-IgG (generated against the last 12 residues of SNAP-25) and anti-Sbr/Cbr-IgG (Lawrence *et al.*, 1996). Bound antibodies were detected indirectly using anti-species-specific IgGs-covalently conjugated to horseradish peroxidase and visualised by the enhanced chemiluminesence (ECL)-detection system.

Assessment of the susceptibilities of bacterially-expressed wild-type and mutant SNAP-25 to cleavage by BoNT/A using a novel ELISA. Purified full-length GST-SNAP-25 (Smith & Johnson, 1988) was coated on 96-well plates, rinsed, blocked and exposed to various concentrations of DTT-reduced BoNT/A at 37 °C for the periods specified in the Figure legends. The wells were then aspirated, washed and probed with anti-SNAP-25(C-terminus)-IgG (Lawrence *et al.*, 1996) or anti-$SNAP\text{-}25_A$-pAb. Unbound IgGs were removed by rapid washing and the primary IgGs bound detected indirectly, using anti-species-specific IgGs-covalently conjugated to alkaline phosphatase, and visualised by colour development upon addition of para-nitrophenyl phosphate substrate. The A405nm values recorded from toxin-treated wells were expressed as percentages of toxin-free control values and plotted against the toxin concentration used. Standard curves relating amounts of intact SNAP-25 remaining in wells were constructed using defined mixtures of full-length or BoNT/A-truncated GST-SNAP-25. The A405nm readings recorded were expressed as percentages of that recorded for the 100% intact protein sample and plotted against the amounts of intact GST-SNAP-25 coated initially. Generally, between 60 and 70 % of the maximal A405nm signal recorded represented 50 % intact SNAP-25 in wells (depending on the primary Ig used).

Alternatively, a capture ELISA was employed to determine the initial rates of BoNT/A cleavage of GST-SNAP-25 at a range of substrate concentrations. GST-SNAP-25

concentrations (0.118 - 15 μM) in KGEP buffer pH7.2 (buffer detailed in Lawrence *et al.*, 1996) containing 10 μg/ml bovine serum albumin (BSA) and 50 μM $ZnCl_2$ were incubated in the absence or presence of 0.05 nM reduced BoNT/A for several time periods chosen to yield less than 15 % cleavage. Reactions were quenched by adjusting pH to 10 and by addition of 20 mM EDTA using a suitable buffer. Next, each GST-SNAP-25 sample was diluted to the same concentration (4 μg/ml) and coated onto multiple wells of a 96-well plate. In addition, mixtures of full-length and BoNT/A-truncated SNAP-25 ($SNAP\text{-}25_A$) were also coated onto wells in order to generate a standard curve (see below). After incubation of wells with the Ig that only recognizes $SNAP\text{-}25_A$ (i.e. anti-SNAP-25A-pAb), unbound IgG was removed by washing and bound Ig was detected using horseradish peroxidase-conjugated anti-species Ig. After washing, bound secondary Ig was visualised using a colour reaction that was measured at 405 nm. Standard curves relating % of $SNAP\text{-}25_A$ of total SNAP-25 protein coated were plotted against the $A_{405\ nm}$ values recorded per well. A linear correlation was observed between increases in absorbance and the % of $SNAP\text{-}25_A$ coated; thus, the % $SNAP\text{-}25_A$ present in the wells of unknown samples could be extrapolated and used to calculate initial rates.

RESULTS AND DISCUSSION

Production of mutant SNAP-25 highly resistant to proteolysis by BoNT/A: arginine at position 198 is crucial for efficient cleavage. A panel of mutants of full-length SNAP-25 (detailed in Figure 1), generated as described in the 1st annual report, was examined for susceptibility to BoNT/A protease relative to the wild-type recombinant protein - the most efficient substrate. Earlier studies (Schmidt and Bostain, 1997) examining the importance of SNAP-25 residues P5 through to P′5 for BoNT/A protease found that the P`1 arginine residue at position 198 residue is critical. Thus, appropriate nucleotide bases in the SNAP-25b gene were altered by PCR-based site-directed mutagenesis and mutant proteins expressed and purified as GST-linked products (see 1st annual report). Eleven different mutated SNAP-25 polypeptides were generated containing either single, double or triple substitutions at P2 through to P′2 positions (listed in Figure 1B).

ELISAs which allow one to quantify the amounts of either full-length SNAP-25 or the BoNT/A-truncated product (residues 1-197; termed SNAP-25_A) were optimised and standardised for measuring proteolysis of recombinant SNAP-25 by this toxin. Either wild-type (wt) or mutant GST SNAP-25s were coated at equimolar amounts onto wells (detailed in Materials and Methods). After blocking excess binding sites with BSA, the wells were exposed to increasing concentrations of reduced BoNT/A; the extents of proteolysis were monitored with either anti-SNAP-25-(C-terminus) reactive antibody (Figure 2A,B) or anti-SNAP-25_A-pAb (Figure 3), depending upon the particular mutant being studied. Use of different antibodies for detection was necessary because alteration of some residues in SNAP-25 protein abolished primary antibody binding. Notably, SNAP-25_{1-202}, A199L, and SNAP-25_{ADD} mutants were not reactive with anti-SNAP-25-(C-terminus)-Ig. Conversely, the numerous mutants described that contained substitutions at position Q197 did not react with anti-SNAP-25_A-pAb after BoNT/A proteolysis due to the loss of this essential epitope (detailed in the 1st annual report). Therefore, anti-SNAP-25-(C-terminus) Ig was used to detect the toxin's cleavage of single mutants altered at the P1 and/or P′1 sites (Figure 2A,B). The relative amounts of wt or mutant SNAP-25s remaining intact following toxin treatment are expressed as A405 nm readings relative to those for toxin-free controls. Incubation of SNAP-25 or its P1/P′1 variants for 6 h at 37°C with very high concentrations of reduced BoNT/A (up to 450 nM) was required to give partial or extensive proteolysis of the single- and double-point mutants. BoNT/A proteolysis of the three remaining SNAP-25 mutants that lacked reactivity to anti-SNAP-25-(C-terminus) Ig, were assessed by ELISA using anti-

$SNAP\text{-}25_A$-pAb (Figure 3). As before, wells coated with equal amounts of either wt or mutant SNAP-25 were exposed to increasing concentrations of reduced BoNT/A (up to 100 nM) for 90 min at 37°C. After development and subtraction of the A405 nm signal in wells lacking SNAP-25 from those for all samples, the resultant values (means ± S.D.; n ± 4) were plotted. Notably, wt and SNAP-25 mutants all exhibited different susceptibilities to BoNT/A (Figure 2 and 3). The EC_{50} for the cleavage of wt and each SNAP-25 mutant by BoNT/A was extrapolated from Figure 2 and 3 and displayed in Table 1.

It was found that changes at the P'1, but not at the P1 position, yielded proteins highly resistant to BoNT/A compared to the wild-type substrate; replacement of P′1 arginine with either alanine (R198A) or threonine (R198T) reduced degradation of SNAP-25 by ~ 560- and 16,000-fold, respectively (Table 1). In contrast, alteration of the P1 glutamine to alanine caused little change (Q197A; Table 1). Similar observations highlighting the importance of P`1 substrate residues has also been reported by Shone & Roberts (1994) in the case of BoNT/B cleavage of Sbr peptides. Notably, they also observed that alterations of the nature of the residue at the P1 position in Sbr is less important for BoNT/B proteolytic activity, as found herein for SNAP-25 and type A activity. The rate of hydrolysis by BoNT/A was also strongly affected by substitutions at the P′2 position; changing an Ala to a larger hydrophobic Leu residue caused ~50-fold lower susceptibility to BoNT/A-mediated cleavage. Interestingly, Schmidt & Bostian (1997) reported that when an analogous replacement was made in their small substrate, the mutant was capable of binding BoNT/A as efficiently as the wild-type substrate. However, our observation that the A199L mutant is significantly proteolysed by toxin would appear to exclude its usefulness as an inhibitor. On the other hand, the double-point mutations incorporating the innocuous P1 alanine replacement, in addition to alterations at the P′1 site, to either alanine (Q197A/R198A), lysine (Q197A/R198K) or glutamic acid (Q197A/R198E) produced even lower susceptibilities to proteolysis by type A toxin (~4,400-, 38,000- and 38,000-fold, respectively). Unexpectedly, the double-point SNAP-25 mutant Q197A/R198A which shared the Gln to Ala substitution at the P1 position (a single mutation that has little effect on BoNT/A activity alone) but that also includes an Ala substitution at the P′1 residue, was much more resistant to the toxin (i.e. approximately 4400-fold lower susceptibility to BoNT/A protease) compared to the single P′1 mutant containing the R198A alteration (i.e. ~560-fold slower). The underlying reason for this anomaly is uncertain, but may reflect a conformational change in the substrate at the cleavage site. Therefore, point mutations may significantly alter the conformation of the C-terminus of SNAP-25, as far as this protease is concerned. Other double-point mutations at

the P1 and P′1 positions [i.e. the Q197/R198 sequence to either KH [Q197K/R198H; naturally occurring in Torpedo SNAP-25 (Washbourne *et al.*, 1997) or WW (Q197W/R198W)] caused far less resistance to BoNT/A (~ 440- and 96-fold, respectively; Table 1). The relative susceptibility of the Torpedo form of SNAP-25 to BoNT/A was examined because its electric organ is a strictly cholinergic tissue that serves as a useful experimental model and shares many similarities to the mammalian NMJ. The unexpectedly modest reduction in susceptibility observed upon changing the P1 and P′1 positions in SNAP-25 from Q-R to W-W suggests that the BoNT/A enzyme S`1 sub-site favours aromatic residues to some extent. In order to confirm this possibility, extra experiments are required involving single point P`1 mutations of the wt Arg to either Trp, Phe or Tyr. During the course of this study, others (Vaidyanathan *et al.*, 1999) reported a similar high resistance of SNAP-25 to scission by BoNT/A upon incorporation of P′1 replacements.

In addition to the above changes, other novel changes have been inserted into the SNAP-25 protein. Whilst removal of four residues resulted in only minor alteration in susceptibility to the protease, addition of 18 residues had a moderate inhibitory effect. In the latter case, addition of 18 amino acids to the C-terminus of SNAP-25 (sequence added, SEFHRDSLTICLARFGDD; derived from the pGEX vector) reduced susceptibility to BoNT/A proteolysis by ~7-fold. Based on this information, the possibility exists of adding a seven amino acid C-terminal biotin-mimic tag (available commercially) to SNAP-25 enabling direct measurement of BoNT/A cleavage with enzyme-linked streptavidin. Development of such an assay would provide a more rapid assay compared to the indirect two-step antibody-based approach currently being employed, and eliminate the need to generate highly specific Ig preparations.

The lack of immunoreactivities of both triple mutants towards either anti-SNAP-25-(C-terminus) Ig or anti-SNAP-25_A-Ig precluded the ELISA-based assessment of their susceptibilities to proteolytic attack by BoNT/A. Therefore, their susceptibility to cleavage by BoNT/A was assessed, following a 2 h incubation at 37°C with different concentrations of reduced toxin, by SDS-PAGE and visualisation of protein by silver staining. Whilst the wt substrate was completely proteolysed by the lowest concentration of BoNT/A tested (Figure 4; 0.1 nM), the small shift in Mr seen with the latter was not detectable for either N196Q/Q197A/R198/K (Figure 4B) or Q197A/R198K/A199L mutants (Figure 4C) when up to 100 nM toxin was used. Although the latter method is prone to possible errors, it appears that these triple mutants are also highly resistant to attack by the toxin.

BoNT/A proteolyses full-sized SNAP-25 more efficiently than small peptide substrates: co-incubation with S4-motif peptides does not improve protease activity. In pursuit of a minimal-sized efficient substrate of BoNT/A on which to base the development of inhibitors, it was important to compare the initial rate (v_o) of proteolysis measured for the recombinant full-sized SNAP-25 with those obtained for smaller synthetic substrates. For this purpose, BoNT cleavage of different GST-SNAP-25 concentrations (0.118 to 15μM) in solution were measured via an ELISA, standardised with defined mixtures of BoNT/A-truncated and intact protein detected using anti-SNAP-25_A-pAb (see Materials and Methods). At the highest GST-SNAP-25 concentration (15 μM), BoNT/A gave a v_o of 12.6 ± 1.1 μmoles/min/mg toxin (mean ± S.D.). Analysis of the results using the Lineweaver-Burke plot revealed that saturation of the enzymes active site had not been achieved at the maximum substrate concentration employed (Figure 5). Therefore, both K_m and V_{max} values were unobtainable. The HPLC assay of Schmidt was employed to assess the kinetic parameters of two small synthetic peptide substrates; residues 187-203 (i.e. the substrate optimised by Schmidt and Bostain, 1995; termed acetylated NH 187–203 CA) or a 26-mer peptide residues 181-206 (detailed in 1st annual report; also see Figure 6). The kinetic parameters recorded for these three substrates are displayed in Table 2. Both of these short peptides were proteolysed by BoNT/A at initial rates very substantially lower than that observed above for full-length SNAP-25. Previous studies performed by Schmidt and Bostian (1995, 1997) found that this 17-amino acid peptide served as a substrate for BoNT/A; however, extending its length to 66 residues gave much better cleavage (Washborne *et al.*, 1997). Thus, it appears that a 9 residue SNARE motif (145-153), located far upstream of the scissile bond of type A (Rossetto *et al.*, 1994), is essential for efficient recognition by the toxin.

Although 17-mer C-terminal SNAP-25 and the 26-mer (shown herein) are poorly proteolysed by BoNT/A relative to the full-length substrate, the former provides a convenient model for investigating the residues required on either side of the scissile bond. Moreover, site-modified synthetic peptides have the added advantages that they are often much more soluble than large recombinant proteins, allowing use of sufficiently high concentrations to determine the effects of the above modifications on enzymic kinetic parameters. Unfortunately, the apparent low affinities of BoNTs for their optimal substrates (Km between 0.1-2 mM; TeTx, Cornille *et al.*, 1994; TeTx and BoNT/B, Foran *et al.*, 1994) preclude the usefulness of bacterially-expressed recombinant substrates because of their limited solubility. When stored, as GST-fusion proteins or with GST removed, at

concentrations in excess of 1 to 2mg/ml protein (equivalent to 40 μM), association/aggregation leads to their precipitation at neutral pH and temperatures ≥ 23°C (unpublished data). Also, the isolated soluble recombinant SNAP-25 molecule forms homo-oligmers ≥ 6-mers at 0.1 mg/ml concentration (Dr. P. Foran, unpublished observation; Fasshauer *et al.*, 1997).

In view of this discrepancy between the rates of cleavage of the full-length protein and small synthetic peptides, the S4 SNARE motif was evaluated for ability to improve this proteolysis by analogy with TeTx and Sbr domains (Cornille *et al.*, 1997). Unfortunately, a surrogate peptide (acetylated–NH-ARENEMDENLEQVSG-$CONH_2$) encompassing the S4 SNARE motif of SNAP-25 (see Figure 6) proved ineffective (up to 0.1 mM) in accelerating v_o for BoNT/A proteolyses of either the 26- or 17-mer peptides; at high concentrations, each was inhibitory (IC_{50} ~ 2 mM). Future work will examine if the segment of bacterially-expressed GST-SNAP-25 (residues 1-180), homologous to the BoNT/E-cleaved product, can potentiate the activity of BoNT/A protease towards small substrates because of its S4 SNARE motif being present in the optimal conformation. In addition, the potential inhibitory effects of the S4-SNARE motif peptide on BoNT/A cleavage of the full-sized SNAP-25 was assessed by ELISA; it inhibited BoNT/A proteolysis of full-sized SNAP-25 with an IC_{50} of approximately 0.5 mM (Figure 6).

Different time courses for recovery of evoked secretion from poisoning with BoNT/A or B: a process coincident with reappearance of intact SNARE. To investigate the important question of how BoNTs exert their inhibitory actions for prolonged periods, neuroendocrine adreno-chromaffin cells were intoxicated for 24 h with BoNT/A (6.6 nM) or B (66 nM) using a low-ionic strength medium (LISM), to overcome the absence of high affinity acceptors (Lawrence *et al.*, 1996). Evoked secretion and SNARE contents were assessed at regular intervals on days 5, 19, 40 and 56, post intoxication (Figure 7). Ba^{2+} was employed instead of other common stimuli (e.g. 55 mM K^+ or nicotine that induce Ca^{2+} entry) because it evokes catecholamine release up to 50% of the total content compared to only ~20 % by the latter stimuli, while still exhibiting the same SNAP-25 and Sbr/Cbr requirements as that induced by Ca^{2+} (Lawrence *et al.*, 1996). As expected, BoNT/A or /B treatments at day 5 post-intoxication both yielded extensive inhibition of Ba^{2+}-evoked catecholamine release of 84 ± 0.7% and 90 ± 0.4% (means ± S.E.; n=4), respectively, relative to that for toxin-free controls. Intact SNAP-25 was monitored using anti-SNAP-25(C-terminus)-IgG, whilst intact Sbr and Cbr (a close BoNT/B-sensitive homologue (McMahon *et*

al., 1993) was assessed using anti-Sbr/Cbr-IgG. Both of these antibody preparations were un-reactive towards the BoNT-cleaved products. Further, the presence of BoNT/A-truncated SNAP-25 was assessed using anti-SNAP-25_A-pAb. This antibody was unreactive with intact SNAP-25 in neurotoxin-free cells, but reacted strongly with the truncated product in BoNT/A poisoned cells (Figure 7; and as found previously by Lawrence *et al.*, (1997). The BoNT-mediated inhibition of secretion noted at day 5 was accompanied by total or near-complete cleavage of intact SNAP-25 or Sbr/Cbr (Figure 7). In addition, the BoNT/A-truncated SNAP-25 was present at this time (Figure 7A). At day 40 post-intoxication, a trace of intact SNAP-25 was observed in BoNT/A-poisoned cells; however, the amount noted was much lower than normal and insufficient to significantly influence the level of evoked secretion recorded compared to day 5 (Figure 7A). However, at day 56 post-intoxication, slightly larger amounts of intact SNAP-25 were noted in BoNT/A poisoned cells, apparently enough to cause a statistically significant, but partial, recovery of exocytosis, equivalent to a doubling of the amount of Ba^{2+} evoked catecholamine release from 16 to 34%. Throughout this time course, $SNAP\text{-}25_A$ persisted in intoxicated cells (Figure 7A). Difficulties inherent to primary cell cultures precluded studies longer than eight weeks post-intoxication, periods that would appear to be necessary to attain complete recovery from BoNT/A.

Conversely, 56 days was sufficient to allow near-complete recovery from BoNT/B-poisoning. The inhibition of evoked secretion in BoNT/B-poisoned cells initially noted on day 5 post-intoxication of 90 ± 0.4%, gradually subsided until only 10 ± 3.4% inhibition (means ± S.E.; n=4) remained at day 56. Recovery of evoked secretion in BoNT/B-treated cells was coincident with reappearance of intact Sbr/Cbr to a level comparable to that observed in toxin-free cells (Figure 7B).

These different recovery periods for exocytosis in BoNT/A- and B- poisoned chromaffin cells shows striking similarity with previously published data found after focal intramuscular injection of either toxin in humans (Sloop *et al.*, 1997). Here, it is shown for the first time that the near-complete recovery after BoNT/B poisoning is due to appearance of intact Sbr/Cbr. Notably, the time course for the latter correlates with that found for chromaffin cells poisoned with TeTx (Bartels *et al.*, 1994), which suggests that either the turnover of BoNT/B and TeTx are very similar and/or the rate of replenishment of intact Sbr/Cbr limits the resumption of catecholamine release. The much slower rates for recovery, reappearance of newly-synthesised intact SNAP-25, and disappearance of the truncated product in BoNT/A-treated cells show that either this serotype remains active for longer than BoNT/B and/or the turnover of SNAP-25 is significantly slower than that of Sbr/Cbr.

Expression of wild-type and mutant SNAP-25 in CHO cells. Correct expression of recombinant SNAP-25 from the pcDNA1.1/Amp-SNAP-25 constructs was assessed in CHO cells, since they lack endogenous SNAP-25, using immunoblotting. Anti-SNAP-25(recombinant)-IgG, which recognises both the full-length and BoNT-truncated SNAP-25s, detected expression of SNAP-25 in cells transfected with the wild-type, mutant R198T or SNAP-25_A mammalian expression constructs (Figure 8). Moreover, recombinant SNAP-25 proteins exhibited the appropriate Mr compared to the native protein present chromaffin cells (Figure 8). Notably, both wild-type and R198T SNAP-25 proteins from toxin-free cells reacted with anti-SNAP-25(C-terminus)-IgG unlike SNAP-25_A; instead, the latter was solely recognised by anti-SNAP-25_A-pAb (Figure 8). In addition, mammalian-expressed SNAP-25s exhibited equivalent sensitivities to BoNT/A protease as bacterial-generated proteins. Immunoblotting using anti-SNAP-25(C-terminus)-IgG revealed proteolyses of the majority of the wild-type by BoNT/A (leading to the appearance of the SNAP-25_A immunoreactivity), whilst the protease-resistant SNAP-25 R198T mutants reactivity was not altered (Figure 8). Notably, both wt and R198T mutant SNAP-25s were expressed at equivalent levels in transfected cells, using the same transfection protocol. As expected, immunoreactivity for SNAP-25_A in CHO cells was unaffected by BoNT/A treatment (Figure 8). Having demonstrated that this SNAP-25 mutant was resistant to BoNT/A cleavage in mammalian cells, plasmids encoding wild-type and mutant SNAP-25 were introduced into chromaffin cells.

Effects of expression of SNAP-25 on evoked exocytosis from control or BoNT/A-pre-treated chromaffin cells. It was considered a possibility that introduction of SNAP-25 into BoNT/A-poisoned cells should restore regulated exocytosis by replacement of the endogenous SNAP-25_A. To investigate the effects of introduced SNAP-25 on evoked secretory function in chromaffin cells, the protein was transiently co-expressed with hGH. The latter, which is normally absent from cells, co-localises with catecholamines in LDCVs (Holz *et al.*, 1995). Thus, evoked hGH secretion serves as an excellent reporter for LDCV exocytosis (Figure 9). In control or BoNT/A-treated cells that had been transfected with vectors encoding hGH, and the non-toxic protein CAT, similar amounts of Ba^{2+}-evoked hGH and catecholamine secretion were observed (Figure 7, 9A). Transfection with a plasmid encoding SNAP-25 did not alter the amount of hGH secreted from toxin-free cells; significantly, over-expressed SNAP-25 was unable to overcome the extent of inhibition due

to BoNT/A poisoning (Figure 9A). These results suggested that the persistence of BoNT/A maintained the blockade of release.

Effects of expression of SNAP-25$_A$ on evoked exocytosis from control or BoNT/A-pre-treated chromaffin cells. Having established that wild-type or mutant SNAP-25 are properly expressed, it was deemed important to determine whether the introduced SNAP-25 was participating in exocytosis. Importantly, expression of SNAP-25$_A$ (i.e. analogous to the BoNT/A-cleaved product) in toxin-free cells inhibited evoked hGH secretion by > 90% of control in a manner similar to the BoNT/A pre-treatment. Expression of SNAP-25$_A$ in BoNT/A pre-poisoned cells consistently abolished the residual evoked secretion (~ 10 % of total) that commonly remained following toxin treatment (Figure 9B). Thus, not only does introduced SNAP-25$_A$ replace the endogenous protein, the displacement of the intact SNAP-25 with the BoNT/A-truncated product establishes that BoNT/A poisoning is due solely to cleavage of SNAP-25.

Rescue of evoked exocytosis in BoNT/A-pretreated chromaffin cells by expression of SNAP-25 mutants resistant to the toxin : validation of the recovered secretion by its inhibition with BoNT/E. Having established the validity of the system, transfection of plasmids directing the expression of BoNT/A protease-resistant SNAP-25s were performed. Introduction of SNAP-25 mutants into toxin-free chromaffin cells caused only a small reduction in the ability to secrete hGH, as compared to controls (Figure 9A). As hoped, expression of each protease-resistant SNAP-25 in BoNT/A-treated cells (in which catecholamine release is inhibited; data not shown) almost completely rescued secretory function close to the levels recorded for toxin-free controls (Figure 9A). This experiment was repeated twice, with the pattern being the same in each case. Overall, we have established that expression of BoNT/A protease-resistant SNAP-25s can efficiently rescue secretory function in cells poisoned 5 days earlier.

An additional experiment was performed to conclusively demonstrate that the rescue of evoked hGH secretion in BoNT/A pre-intoxicated cells was a direct result of the participation of expressed BoNT/A protease-resistant SNAP-25. Digitonin-permeabilisation was used to introduce BoNT/E into cells (because the LISM protocol is ineffective for BoNT/E), so that this toxin could further proteolyse the artificially-expressed BoNT/A protease-resistant SNAP-25 R198T mutant, thus, negating its protective effect. As expected in cells lacking protease-resistant SNAP-25, prior BoNT/A poisoning caused an 82 ± 4.3% inhibition of hGH

release when compared to toxin-free cells. On the other hand, evoked hGH secretion in BoNT/A pre-intoxicated cells that expressed the SNAP-25 protease-resistant R198T mutant was largely protected. Importantly, when BoNT/A-pre-treated cells expressing R198T mutant were permeabilised and exposed to reduced BoNT/E (using conditions known to proteolyse nearly all SNAP-25 (Lawrence *et al.*, 1997), the partial rescue of evoked secretion in BoNT/A-poisoned cells (noted above) was largely abolished, and an 88 ± 7.8% inhibition of evoked hGH secretion was recorded (Figure 9B). This experiment was repeated twice, with the pattern being the same in each case.

The ability of BoNT/A-resistant SNAP-25 to rescue release provides some important insights about SNAP-25 and its BoNT/A cleavage site. First, over-expression of either wild-type SNAP-25 or BoNT/A-resistant mutants do not seem to cause toxicity to the cell, since the characteristics of release from the toxin-free cells appear to be unaffected. In addition, recombinant SNAP-25 has been previously shown to be targeted to the appropriate plasma membrane locations in PC12, as well as insulinoma cells (Bark *et al.*, 1995; Gonelle-Gispert *et al.*, 1999). As SNAP-25 must complex with the limited amounts of syntaxin1 and Sbr present in order to exert its co-operative exocytotic function, it is reasonable to assume that most of the excess protease-resistant SNAP-25s competes out the native SNAP-25 for SNARE complex formation. The fact that BoNT/E inhibits this rescued exocytosis provides further proof for the participation of BoNT/A-resistant SNAP-25 in SNARE-mediated exocytosis. Realising these important considerations, it is evident that residues Q197 and R198 in SNAP-25, which were altered in the protease-resistant mutants, are not essential for evoked exocytosis. These studies and those of others have shown that there is a high degree of amino acid degeneracy in SNAP-25. When the BoNT/E cleavage site (residues 180-181) in SNAP-25 was mutated, it was found that over-expression of BoNT/E-resistant SNAP-25 prevented the anticipated inhibition of evoked secretion upon exposure to BoNT/E (Gonelle-Gispert *et al.*, 1999), which suggests that these residues are also not critical for exocytosis. Further, a recent site-directed mutagenesis study of SNAP-25 has revealed that mutations of hydrophobic residues, found within the core of the four-helix bundle, significantly limited rescue after BoNT/E poisoning (Chen *et al.*, 1999). The full extent of this degeneracy can be seen when human SNAP-23 (a BoNT/E-resistant non-neuronal homologue which is only ~60 % identical to SNAP-25) is expressed in permeabilised insulinoma cells; this supported the same extent of exocytosis upon treatment with BoNT/E as that found for control cells (Saduol *et al.*, 1997). However, our study is the first to report the amino acid degeneracy of SNAP-25

by quantifying the abilities of BoNT/A-resistant mutants to rescue exocytosis in intact BoNT pre-poisoned cells

Three weeks after exposure to BoNT/A, only protease-resistant SNAP-25 rescues regulated exocytosis : demonstration of the persistence of active toxin protease. The inability to achieve rescue of exocytosis upon transfection of wild-type SNAP-25 into BoNT/A-poisoned cells suggested that the activity of the toxin persisted for at least a week in these cells (Figure 9A). To determine how long BoNT/A remained active, this experiment was repeated up to 3 weeks after exposure to BoNT/A (Figure 10). As expected, toxin-free cells transfected with hGH and CAT encoding control plasmids (i.e. recombinant SNAP-25-free control), released large amounts of hGH in a divalent cation-dependent manner (Figure 10). Cells exposed to BoNT/A 3 weeks previously, when transfected with the same vectors gave a much reduced amount of evoked secretion (Figure 10). Therefore, toxin pre-treatment still afforded a large extent of blockade even after three weeks. As before, expression of wild-type SNAP-25 did not significantly alter the level of evoked hGH secretion occurring in toxin-free cells and, significantly, failed to overcome the blockade in toxin-treated cells (Figure 10). It seems likely that the newly-expressed wild-type SNAP-25 was cleaved by active BoNT/A protease persisting in the cells. Importantly, the extent of evoked hGH secretion recorded from cells intoxicated three week earlier expressing protease-resistant SNAP-25 R198T was returned to the normal level being comparable to that occurring from toxin-free cells expressing either the same mutant or CAT (see above; Figure 10). This experiment was repeated four times, with the pattern being the same in each case. Hence, we show for the first time that BoNT/A is proteolytically active for at least three weeks after poisoning. This prolonged activity suggests that, at least in chromaffin cells, the continued activity of BoNT/A-LC plays a key role in the continued inhibition of release. Moreover, it is likely that toxic amounts of active BoNT/A protease remain in cells even 56 days post-exposure and continues to do so until the eventual complete recovery of evoked secretory function (not determined). Whilst this agrees with previous data published on chromaffin cells (Bartels *et al.*, 1994), it seems to contradict some more recent findings. Human muscles at the NMJ double-poisoned by injection with a mixture of BoNT/A and /E recovered as if they had been solely intoxicated with BoNT/E, which has a shorter time-course for recovery than A (Eleopra *et al.*, 1998). These results suggest it is not the continued BoNT/A activity but the persistence of SNAP-25_A that limits recovery. However, it is unclear how the removal of 17 extra amino acids from the C-terminal end of SNAP-25_A leads to a more rapid recovery

of neurotransmission. It is likely that this apparent contradiction between results in chromaffin cells (our data and Bartels *et al.*, 1994) and those found at the NMJ is due to the different relative ratio of SNAP-25 synthesis to BoNT/A-LC activity but this remains to be determined.

KEY RESEARCH ACCOMPLISHMENTS

1. BoNT/A exerts a prolonged intoxication of adreno-chromaffin cells that is accompanied by complete cleavage of SNAP-25 and continues for at least 2 months (the maximum period studied), presumably due to the persistence of cleaved SNAP-25 (residues 1-197).
2. Exocytosis in BoNT/B-treated cells, initially inhibited completely due to cleavage of Sbr and Cbr targets, resumed in full after 2 months; this was accompanied by the appearance of normal amounts of the intact target proteins.
3. The reported time courses for recovery from neuromuscular paralysis following injection of BoNT/A into patients parallels that found herein, validating the chromaffin cell model.
4. BoNT/A proteolyses full-sized SNAP-25 more efficiently (~ 100 fold faster at 15 μM) than smaller peptide substrates (17 or 26 amino acids in length) which lack the S4 motif (residues 145-155).
5. A surrogate peptide encompassing the S4-motif of SNAP-25 proved ineffective in accelerating BoNT/A proteolysis of the above-noted synthetic peptides or full-length SNAP-25.
6. The susceptibilities of 13 different single, double and triple point mutants of SNAP-25 (generated as outlined in 1st annual report) to proteolysis by BoNT/A have been assessed by ELISA.
7. The P′1 arginine of SNAP-25 is the most important for defining efficient cleavage by BoNT/A.
8. Additional double- and triple-point mutations of SNAP-25 raised the resistance to BoNT/A up to ~40,000-fold relative to the wt substrate.
9. BoNT/A poisoning of regulated catecholamine release from neuro-endocrine chromaffin cells (a process that shares many of the properties of neurotransmitter release from neurons) can be quickly and completely rescued through the introduction of any of five BoNT/A-resistant SNAP-25s.
10. As expression of BoNT/A resistant, but not wt SNAP-25, rescues catecholamine release in chromaffin cells that have been poisoned for at least three weeks, the LC protease appears to remain active for prolonged periods.

REPORTABLE OUTCOMES

Publications

O'Sullivan, G. A., Mohammed, N., Foran, P. G., Lawrence, G. W. and Dolly, J. O. Rescue of exocytosis in botulinum toxin A-poisoned chromaffin cells by expression of cleavage-resistant SNAP-25: Identification of the minimal essential C-terminal residues. *J. Biol. Chem.* (submitted).

Foran, P. G., Fletcher, L. M., Oatey, P. B., Mohammed, N., Dolly, J. O. and Tavare, J. M. Protein kinase B stimulates the translocation of GLUT4 but not GLUT1 or transferrin receptor in 3T3-L1 adipocytes by a pathway involving SNAP-23, synaptobrevin-2 and/or cellubrevin. *J. Biol. Chem.* **274**, 28087-28095 (1999).

Dolly, J. O., O'Sullivan, G. A., Mohammed, N., Foran, P. G., Lawrence, G. W., Lisk, G., Meunier, F. A. and dePaiva, A. Features influencing botulinum toxin-induced nerve sprouting and recovery from poisoning. *Movement Disorders*, Nov 1999.

O'Sullivan, G.A., Mohammed, N., Lawrence, G. W., Foran, P. G., Meunier, F. A., Ekong, T. A. N., Sesardic, D. and Dolly, J. O. SNAP(-25)ping out of botulinum neurotoxin A posioning: restoring regulated exocyosis in chromaffin cells. Proc. 10th Symposium on Chromaffin Cell Biology, p162, Aug. 1999.

Presentations

J.O. Dolly 'Botulinum toxins give insights into synaptic remodelling and membrane trafficking' In International symposium on 'Latrotoxin and Secretory Systems', Gaeta, Italy, Oct. 1998.

J.O. Dolly, 'Botulinum toxin A: mechanism of action' In 3rd Congress of the European Federation of Neurological Societies, Seville, Sept 1998

J. O. Dolly, 'Exocytosis probed with botulinum toxins' Birmingham University, Oct. 1998.

J.O. Dolly, 'Neuro-exocytosis probed with botulinum toxins: basis for their clincal usefulness' In Neuroscience Research Symposium, London, July 1999.

Patent

Filed internationally on Aug.1999 entitled 'Rescue of Ca^{2+}-regulated exocytosis after botulinum A posioning by targetted expression of toxin-resistant SNAP-25.

Degree obtained

Dr N. Mohammed awarded Ph.D.

CONCLUSIONS

In summary, the following points should be emphasised. First, in chromaffin cells BoNT/A-LC appears to remain active for at least three weeks, as introduction of wild-type SNAP-25 does not rescue release. Second, BoNT/A poisoning can be rescued through the expression of BoNT/A-resistant SNAP-25. These novel findings establish the proof of principle for fast rescue from BoNT/A intoxication by this innovative but straight forward transfection process. Future work will concentrate on the targeting of effective treatments for botulism to the cholinergic nerve using the non-toxic BoNT/A cholinergic specific transporter. It is envisaged that the latter transporter will either be directly attached to therapeutic molecules or attached to viral particles or liposomes containing the agent to be tested. In addition, a dual strategy will be adopted for effective treatment of botulism involving the use of small inhibitors of BoNT/A protease (currently being successfully developed by Drs J. Schmidt and K. Bostain at USAMRIID) to initially arrest the toxins' activity, prior to replacement of cleaved target protein with intact toxin-resistant SNAP-25 (by direct delivery and/or using gene expression vectors) to rescue neuroexocytosis.

REFERENCES

Bark, I. C., Hahn, K. M., Ryabinin, A. E., and Wilson, M. C. (1995) *Proc. Natl. Acad. Sci. U. S. A.* 92(5), 1510-1514

Bartels, F., Bergel, H., Bigalke, H., Frevert, J., Halpern, J., and Middlebrook, J. (1994) *J. Biol. Chem.* 269(11), 8122-8127

Chen, Y. A., Scales, S. J., Patel, S. M., Doung, Y.-C., and Scheller, R. H. (1999) *Cell* 97, 165-174

Cornille, F., Goudreau, N., Ficheux, D., Niemann, H., and Roques, B. P. (1994) *Eur. J. Biochem. 222*, 173-181.

Cornille, F., Martin, L., Lenoir, C., Cussac, D., Roques, B. P., and Fourniezaluski, M. C. (1997) *J. Biol. Chem. 272*, 3459-3464

dePaiva, A., Meunier, F. A., Molgo, J., Aoki, K. R., and Dolly, J. O. (1999) *Proc. Natl. Acad. Sci. U. S. A.* 96(6), 3200-3205

Eleopra, R., Tugnoli, V., Rossetto, O., De Grandis, D., and Montecucco, C. (1998) *Neurosci. Lett.* 256(3), 135-8

Fasshauer, D., Bruns, D., Shen, B., Jahn, R., and Brunger, A. T. (1997) *J. Biol. Chem. 272*, 4582-4590

Foran, P., Shone, C. C., and Dolly, J. O. (1994) *Biochemistry 33*, 15365-15374.

Foran, P., Lawrence, G. W., Shone, C. C., Foster, K. A., and Dolly, J. O. (1996) *Biochemistry* 35(8), 2630-2636

Gonelle-Gispert, C., Halban, P. A., Niemann, H., Palmer, M., Catsicas, S., and Sadoul, K. (1999) *Biochem. J.* 339(1), 159-165

Holz, R. W., Senter, R. A., and Uhler, M. D. (1995) *Meth. Enzymol.* 257, 221-31

Lawrence, G. W., Foran, P., and Dolly, J. O. (1996) *Eur. J. Biochem.* 236(3), 877-86

Lawrence, G. W., Foran, P., Mohammed, N., DasGupta, B. R., and Dolly, J. O. (1997) *Biochemistry* 36(11), 3061-3067

McMahon, H. T., Ushkaryov, Y. A., Edelmann, L., Link, E., Binz, T., Niemann, H., Jahn, R., and Südhof, T. C. (1993) *Nature* 364(6435), 346-349

Morgan, A., and Burgoyne, R. D. (1997) *Sem. Cell & Dev. Biol.* 8(2), 141-149

Rossetto, O., Schiavo, G., Montecucco, C., Poulain, B., Deloye, F., Lozzi, L., and Shone, C. C. (1994) *Nature 372*, 415-416

Sadoul, K., Berger, A., Niemann, H., Weller, U., Roche, P. A., Klip, A., Trimble, W. S., Regazzi, R., Catsicas, S., and Halban, P. A. (1997) *J. Biol. Chem.* 272(52), 33023-33027

Schechter, I., and Berger, A. (1967) *Biochem. Biophys. Res. Commun.* 27(2), 157-62

Schmidt, J. J., and Bostian, K. A. (1995) *J. Protein Chem. 14*, 703-708.

Schmidt, J. J., and Bostian, K. A. (1997) *J. Protein Chem.* 16(1), 19-26

Shone, C. C., and Roberts, A. K. (1994) *Eur. J. Biochem. 225*, 263-270.

Sloop, R. R., Cole, B. A., and Escutin, R. O. (1997) *Neurology* 48(1), 249-253

Smith, D. B., and Johnson, K. S. (1988) *Gene* 67(1), 31-40

Vaidyanathan, V. V., Yoshino, K., Jahnz, M., Dörries, C., Bade, S., Nauenburg, S., Niemann, H., and Binz, T. (1999) *J. Neurochem.* 72(1), 327-37

Washbourne, P., Pellizzari, R., Baldini, G., Wilson, M. C., and Montecucco, C. (1997) *FEBS Letters 418*, 1-5

Wilson, S. P. (1987) *J. Neurosci. Methods* 19(2), 163-71

APPENDICES

TABLE 1.

The overall susceptibilities of wild-type and various SNAP-25 mutants to cleavage by BoNT/A

SNAP-25 variant	Position of mutated residue(s)	Minimum BoNT/A concentration (nM) necessary to proteolyse 50% of SNAP-25 in the standard assay[a]	Susceptibilities to proteolysis by BoNT/A (relative to wild-type)[b]
Wild-type	None	0.0045 (0.022)	1.0
Q197/R198			
Q197A	P1	0.0054	1.2
R198A	P1′	2.5	560
R198T	P1′	72	16000
A199L	P2′	(1.09)	49
Q197A/R198A	P1, P1′	20	4400
Q197A/R198K	P1, P1′	170	38000
Q197A/R198E	P1, P1′	170	38000
Q197K/R198H	P1, P1′	2.0	440
Q197W/R198W	P1, P1′	0.43	96
Q198A/R199K/ A199L	P2, P1′, P2′	>>100 nM	>>3000
N196Q/Q197A/ R198K	P1, P1′, P2′	>>100 nM	>>3000
$SNAP\text{-}25_{1\text{-}202}$	Removal of 4 residues from C-terminus	(0.031)	1.4
$SNAP\text{-}25_{ADD\ 1\text{-}206\ (+18)}$	Addition of 18 residues to C-terminus	(0.16)	7.3

[a] Standard curves, relating increasing amounts of intact GST-SNAP-25 per well with the increasing A405 nm values recorded using either anti-SNAP-25-(C-terminus)-Ig or, anti-$SNAP\text{-}25_A$-pAb, were used to calculate the A405nm reading which was equivalent to 50 % intact SNAP-25 remaining in wells. These absorbance values were used to extrapolate the concentrations of BoNT/A required to proteolyse 50 % of either the wild-type or mutant SNAP-25 polypeptides from Figure 2 and 3. Whilst incubations with toxin lasted 6 h in experiments shown in Figure 2, in Figure 3 only 90 min incubations were performed; thus, in this case the toxin concentrations necessary for 50 % cleavage of the mutants compared to wt are indicated inside brackets.

[b] Values obtained by dividing the minimal BoNT/A concentration giving 50% cleavage of the SNAP-25 mutant being tested by the minimal concentration which cleaves wild-type; larger values indicate greater resistance to proteolysis.

TABLE 2.

Comparison of the kinetic parameters for BoNT/A cleavage of full-sized SNAP-25, 26- and 17-mer peptides

Substrate	Km (mM)	Vmax (μmoles min^{-1} mg^{-1}	k_{cat} (sec^{-1})	v_o at 15μM (μmoles min^{-1} mg^{-1})	Turnover rate at 15 μM (sec^{-1})
17-mer (NH-187-203CA)*	0.63	3.1	7.7	0.16	0.40
26-mer (residues 181-206)*	0.72	0.5	1.3	0.02	0.06
Full-length@ SNAP-25	>> 0.015	?	?	12.6	31.5

* Assayed using RP-HPLC-based or ELISA@. Incubations with peptide substrates were at 37°C using 10 nM-reduced BoNT/A in 35 mM Hepes pH 7.5 containing 2 mM DTT, 0.8 $mgml^{-1}$ BSA and 1 mM $ZnCl_2$. Alternatively, toxin incubations with full-length GST-SNAP-25 were performed as detailed in the Materials and Methods. Km and Vmax values were obtained from Lineweaver-Burke plots.

FIGURE LEGENDS

Figure 1. Diagrammatic representation of the mutations, deletions and additions to SNAP-25. (A) The C-terminal amino acid sequence (179-206) of SNAP-25 is shown together with the cleavage site of BoNT/A and the residues therein substituted. (B) Schematic of the mutations of the SNAP-25 protein generated as GST fusions and code names [i.e. R198T indicating that arginine at position 198 in the primary amino acid sequence (shown using the single letter code) is changed to threonine].

Figure 2. Assessment by ELISA using anti-SNAP-25-(C-terminus)-Ig of the susceptibilities of wt or mutant GST-linked SNAP-25s to proteolysis by BoNT/A. ELISA plate wells were coated with either wild-type or the specified single (A) or double (B) mutants of SNAP-25 before exposure for 6 h at 37°C to various concentrations of DTT-reduced BoNT/A in KGEP buffer (pH 7.2). The amounts of wild-type or mutant SNAP-25s remaining were probed with anti-SNAP-25-(C-terminus)-Ig. Binding of the latter was quantified using alkaline phosphatase-conjugated anti-species antibodies and a colorimetric assay (as detailed in Materials and Methods). The resultant colour development was measured with an ELISA plate reader. Absorbance values at 405 nm are expressed as percentages of the maximal A405nm values recorded for SNAP-25-containing/toxin-free control wells and are plotted against the corresponding BoNT/A concentrations used. Data are means (± S.D.; $n > 6$).

Figure 3. Measurement by ELISA using anti-SNAP-25_A-pAb of the cleavage by BoNT/A of wt or mutant proteins. GST-linked wt or the mutant SNAP-25 proteins (specified) were coated onto ELISA plate wells at equimolar amounts and various concentrations of pre-reduced BoNT/A were added at 37°C for 90 min in KGEP buffer pH 7.2. The resultant appearance of the $SNAP\text{-}25_A$ molecule was detected by following anti-$SNAP\text{-}25_A$-pAb binding, as outlined in the Figure 2. The resultant absorbancies, measured during a constant development period, are plotted after subtraction of the values for non-toxin treated control wells (means ± SD; $n = 4$).

Figure 4. Assessment of BoNT/A cleavage of wild-type and triple-point mutants of SNAP-25 using SDS-PAGE and protein staining. Reduced BoNT/A at the specified

concentrations was incubated with either 5μM wt GST-SNAP-25 (A), N196Q/Q197A/R198K (B), Q197A/R198K/A199L mutant (C) fusion proteins for 2 h at 37°C in KGEP buffer. Samples were fractionated by SDS-PAGE on 8% acrylamide gels, fixed and proteins were visualised by a silver staining method.

Figure 5. Lineweaver-Burke plot of initial rates of proteolysis by reduced BoNT/A for various concentrations of GST-SNAP-25. A capture ELISA was employed to determine the initial rates of BoNT/A cleavage of GST SNAP-25 at concentrations between 0.118 to 15 μM. (see Material and Methods). Data plotted are means of 16 determinations.

Figure 6. A surrogate peptide encompassing the S4 SNARE motif inhibits BoNT/A cleavage of GST-SNAP-25, 26- and 17-mer peptide substrates. A diagrammatic representation of the SNAP-25 molecule is shown indicating important regions. A S4-motif peptide of the sequence specified was used in experiments where it was pre-incubated for 60 min at 37°C with reduced BoNT/A prior to addition to the substrate specified. Initial rates of cleavage by BoNT/A of the 17- and 26-mer substrates (50 μM) or GST SNAP-25 (0.5 μM) performed at 37°C, in the absence or presence of various concentrations of S4-motif peptide, were assessed by an HPLC assay (analogous to that used by Schmidt and Bostain, 1995, 1997) and ELISA, respectively. The concentrations of the S4-motif peptide necessary to inhibit the initial cleavage rates by 50% for the different substrates are displayed.

Figure 7. Prolonged monitoring of BoNT/A- and /B-induced inhibition of catecholamine release in chromaffin cells and assessment of their SNAP-25 and Sbr/Cbr contents. Intact chromaffin cells were treated for 24 h using a low-ionic strength medium (LISM) in the absence (open bars) or presence of BoNTs (hatched bars) using (A) 6.6nM BoNT/A or (B) 66 nM BoNT/B. Cells were then maintained in culture for up to 56 days by weekly replacement of growth medium. At the specified times, Ba^{2+}-evoked catecholamine release was quantified fluorometrically (means ± S.E.; n=4). After subtraction of basal release values (cells exposed for 15 min to Locke's buffer) from Ba^{2+}-induced release for 15 min exposure to 2mM Ba^{2+} (in Locke's buffer), the evoked component of release was expressed as a percentage of the total cell content. Immediately after measuring secretion, membrane fractions were prepared, as detailed in the Methods, solubilised in SDS, and frozen at –80°C.

At the conclusion of the time course, fractions were subjected to SDS-PAGE followed by immunoblotting with the indicated antibodies.

Figure 8. Expression of wild-type and SNAP-25 mutant in transfected CHO cells. CHO cells, that lack SNAP-25, were transfected with the pcDNA1.1/amp vector incorporating the specified SNAP-25 gene using the Superfect™ reagent. The transfected cells were treated with LISM in the absence or presence of 6.6 nM BoNT/A (indicated), as detailed in Materials and Methods. Two days later, the membrane fraction was isolated from the cells and equal amounts of SDS solubilised proteins were subjected to immunoblotting, using the indicated antibodies. Chromaffin cell membrane fraction was run as a control. The relative amounts of primary antibodies bound were visualised using the methods specified in the legend to Figure7, but only the relevant track positions are shown.

Figure 9. Expression of protease-resistant SNAP-25 mutants, but not wild-type, rescues evoked secretion from BoNT/A-pre-treated cells: sole monitoring of transfected cells using the hGH-based reporter system. (A) Intact chromaffin cells were treated for 24 h with LISM, in the absence (open bars) or presence (hatched bars) of 6.6 nM BoNT/A, as described in the legend to Figure 7. After a 24 h recovery period, the resultant cells were transfected with the pcDNA1.1/Amp mammalian expression vector containing the specified SNAP-25 or CAT gene together with the reporter plasmid encoding human growth hormone (hGH; as outlined in the Materials and Methods). After five days, the hGH secretion only occurring from co-transfected cells under basal and stimulated conditions (using buffers omitting or containing Ba^{2+}), were quantified using a radio-immunoassay (means ± S.E.; n=4). The evoked component of secretion was expressed as a percentage of the measured total cell content. (B) Cells pre-treated with LISM in the absence (open bars) or presence (hatched bars) of 6.6 nM BoNT/A were subsequently transfected with vectors encoding hGH with or without the plasmid encoding the BoNT/A protease-resistant SNAP-25 R198T mutant (indicated), and cultured for an additional five days. These cells were then permeabilised using a digitonin-containing permeabilisation buffer excluding or containing 100 nM of DTT-reduced BoNT/E for 15 min. The Ca^{2+}-evoked component of hGH secretion, measured over the subsequent 15 min period, was quantified (means ± S.E.; n=4) and expressed as a percentage of the total cell content.

Figure 10. BoNT/A proteolytic activity persists in poisoned cells for at least 3 weeks; only protease-resistant SNAP-25 mutants rescue evoked secretion. Intact chromaffin cells were treated for 24 h with LISM, in the absence (open bars) or presence (hatched bars) of 6.6 nM BoNT/A, as described in the legend to Figure 7. After 16 days, the resultant cells were transfected with the pcDNA1.1/Amp mammalian expression vector containing the specified SNAP-25 or CAT gene together with the reporter plasmid encoding hGH, as outlined in the Materials and Methods section. After a a further five days, Ba^{2+}-evoked hGH secretion was assessed (as outlined in the legend to Figure 9).

Figure 1

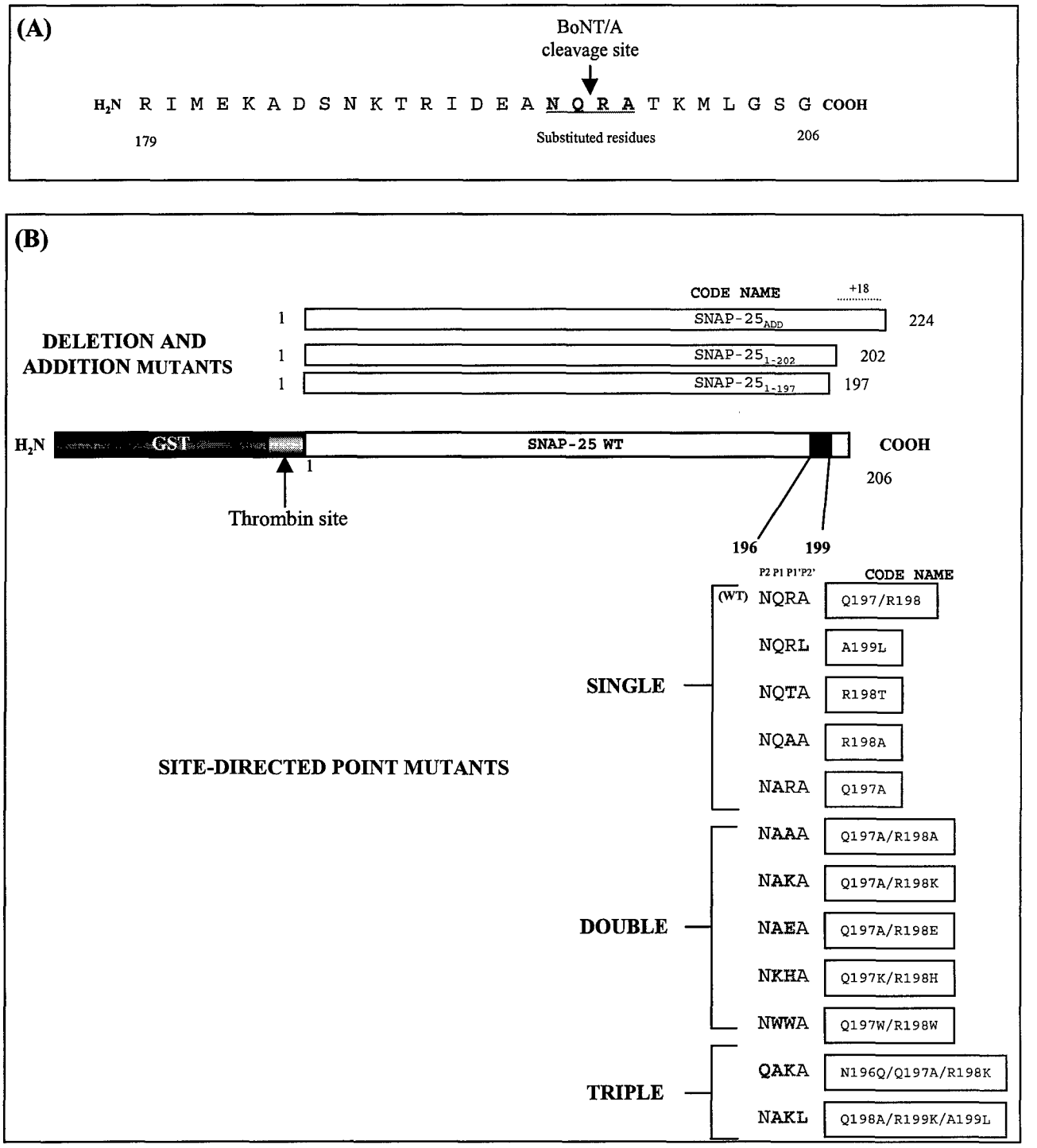
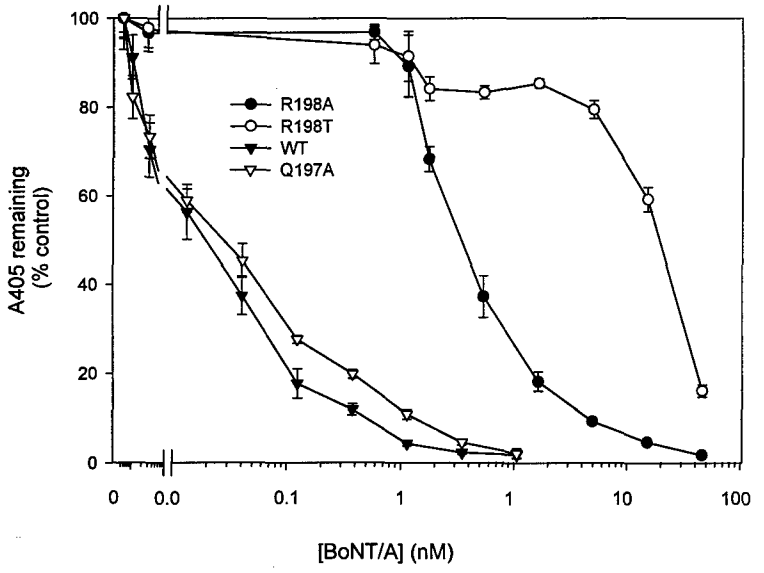

(A)
BoNT/A
cleavage site
H2N R I M E K A D S N K T R I D E A N Q R A T K M L G S G COOH
179
Substituted residues
206
(B)
DELETION AND
ADDITION MUTANTS
CODE NAME
+18
1
SNAP-25ADD
224
1
SNAP-251-202
202
1
SNAP-251-197
197
H2N
GST
SNAP-25 WT
COOH
1
206
Thrombin site
196
199
P2 P1 P1'P2'
CODE NAME
SITE-DIRECTED POINT MUTANTS
SINGLE
(WT) NQRA Q197/R198
NQRL A199L
NQTA R198T
NQAA R198A
NARA Q197A
DOUBLE
NAAA Q197A/R198A
NAKA Q197A/R198K
NAEA Q197A/R198E
NKHA Q197K/R198H
NWWA Q197W/R198W
TRIPLE
QAKA N196Q/Q197A/R198K
NAKL Q198A/R199K/A199L

Figure 2

A


SINGLE POINT
A405 remaining (% control)
100
80
60
40
20
0
R198A
R198T
WT
Q197A
0
0.0
0.1
1
1
10
100
[BoNT/A] (nM)

B

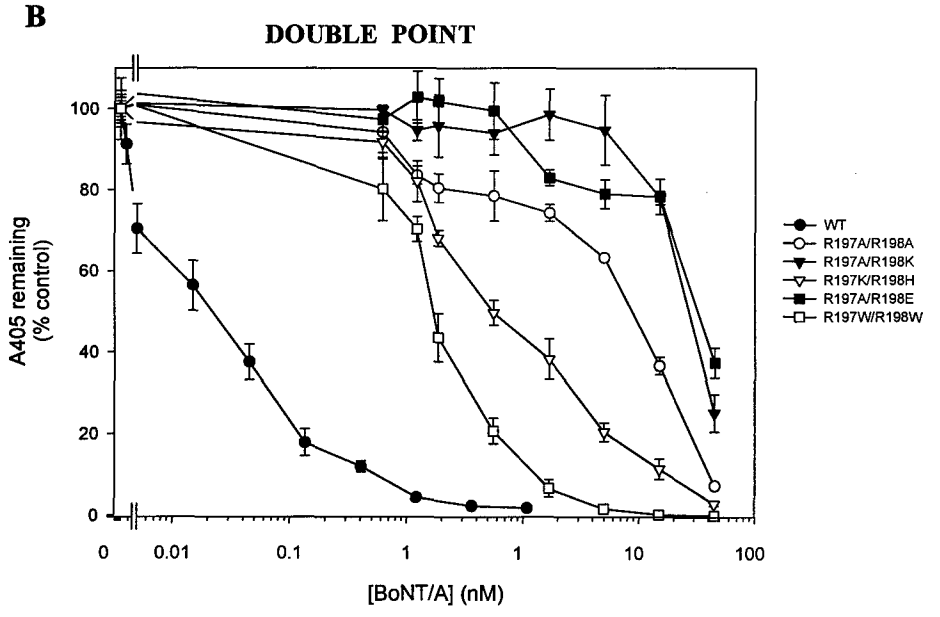

DOUBLE POINT
A405 remaining (% control)
100
80
60
40
20
0
0
0.01
0.1
1
1
10
100
[BoNT/A] (nM)
WT
R197A/R198A
R197A/R198K
R197K/R198H
R197A/R198E
R197W/R198W

Figure 3

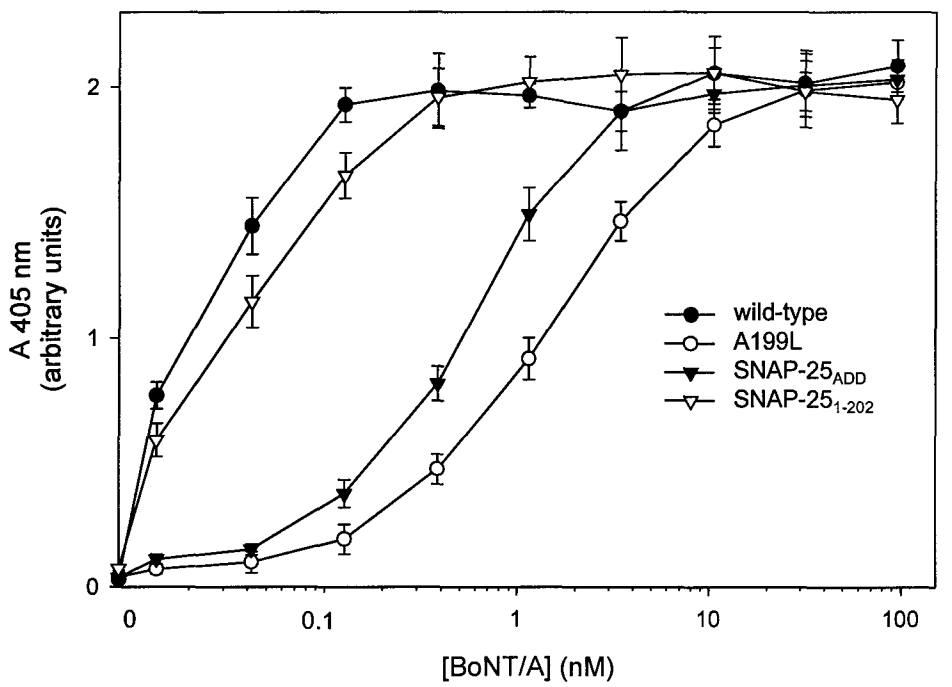

A 405 nm
(arbitrary units)
2
1
0
0
0.1
1
10
100
[BoNT/A] (nM)
wild-type
A199L
SNAP-25ADD
SNAP-251-202

Figure 4

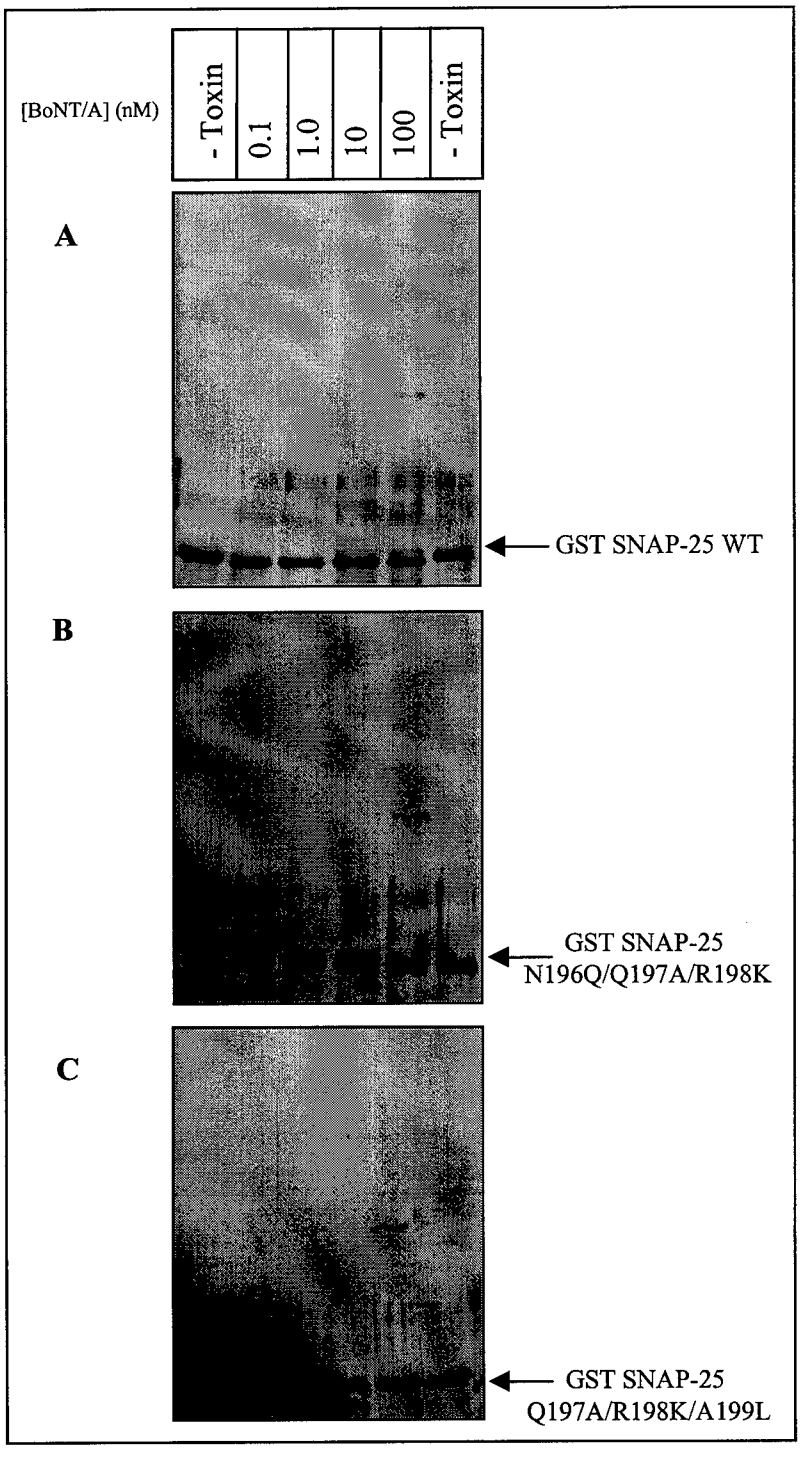

[BoNT/A] (nM)
- Toxin
0.1
1.0
10
100
- Toxin
A
GST SNAP-25 WT
B
GST SNAP-25
N196Q/Q197A/R198K
C
GST SNAP-25
Q197A/R198K/A199L

Figure 5

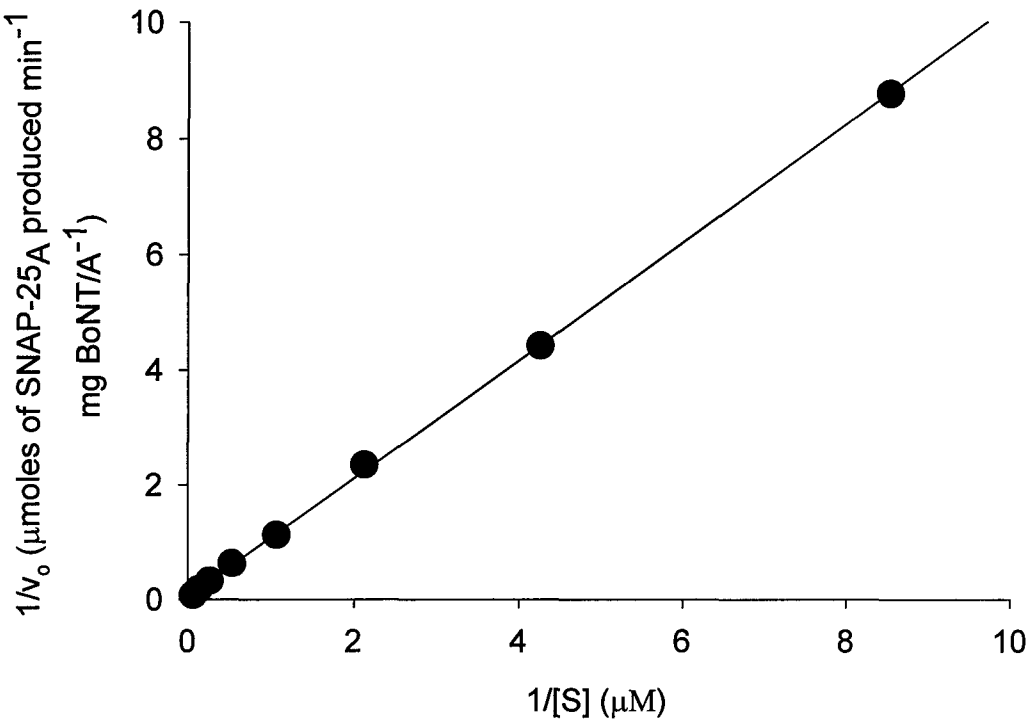
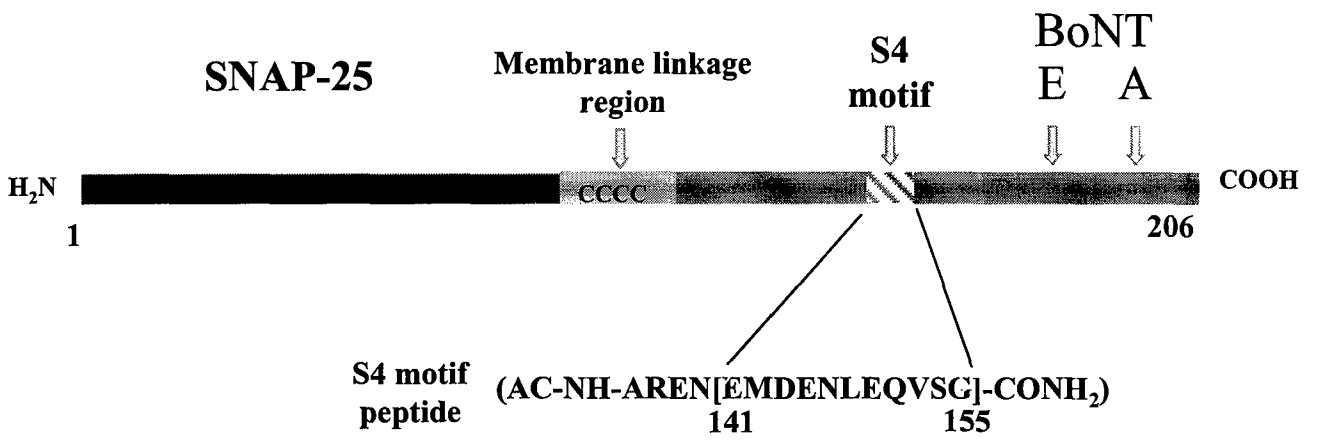

1/v_o (μmoles of SNAP-25$_A$ produced min^{-1} mg BoNT/A^{-1})
10
8
6
4
2
0
0
2
4
6
8
10
1/[S] (μM)

Figure 6



IC_{50} (mM)		
~ 2	17-mer [NH-187-203CA]	Ac- -$CONH_2$ (187 – 203)
~ 2	26-mer substrate	H_2N-Cys- -COOH (181 – 206)
~ 0.5	Full-length SNAP-25	

Figure 7

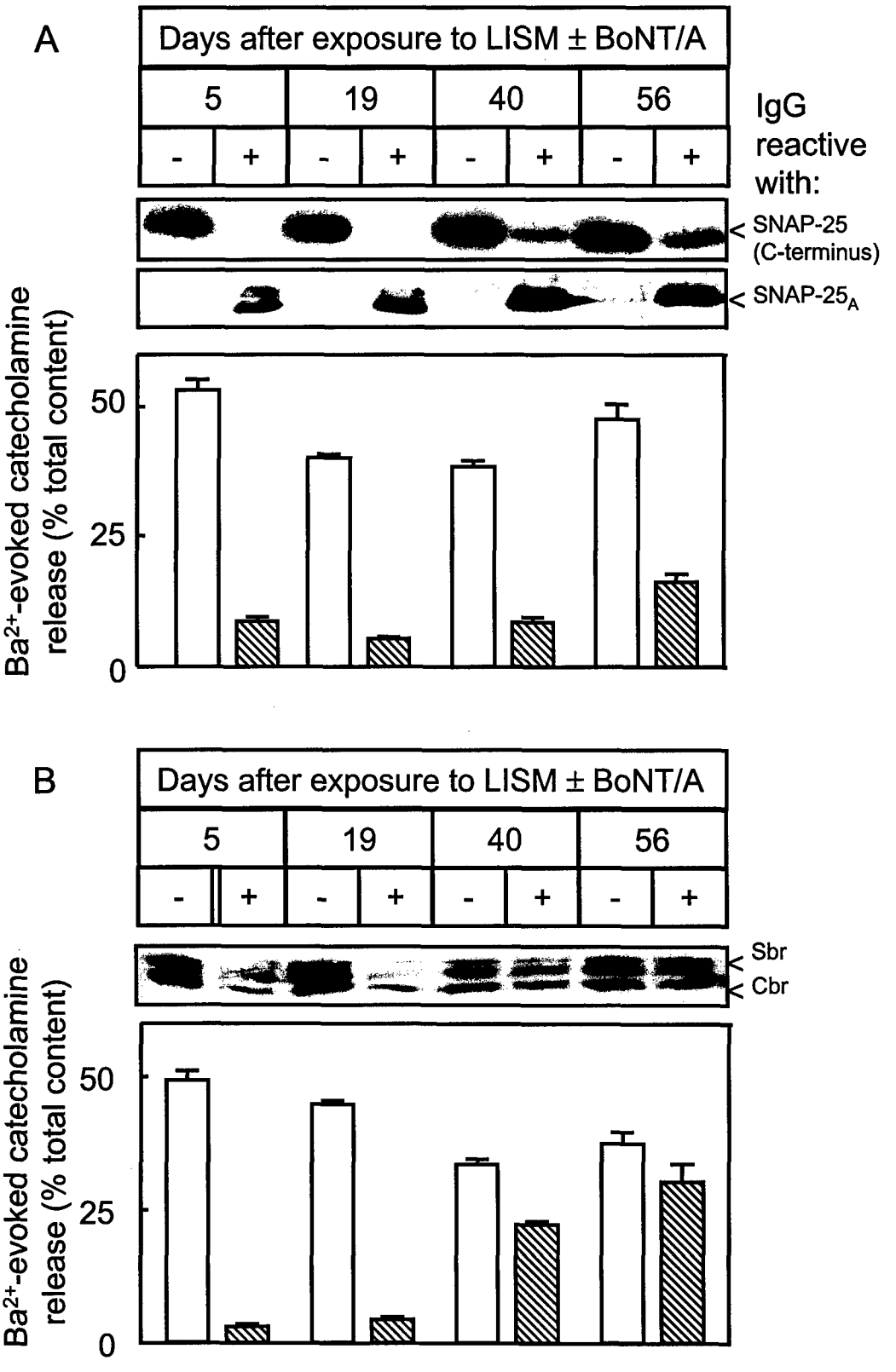

A
Days after exposure to LISM ± BoNT/A
5
19
40
56
- + - + - + - +
IgG reactive with:
SNAP-25 (C-terminus)
SNAP-25A
Ba2+-evoked catecholamine release (% total content)
50
25
0
B
Days after exposure to LISM ± BoNT/A
5
19
40
56
- + - + - + - +
Sbr
Cbr
Ba2+-evoked catecholamine release (% total content)
50
25
0

Figure 8

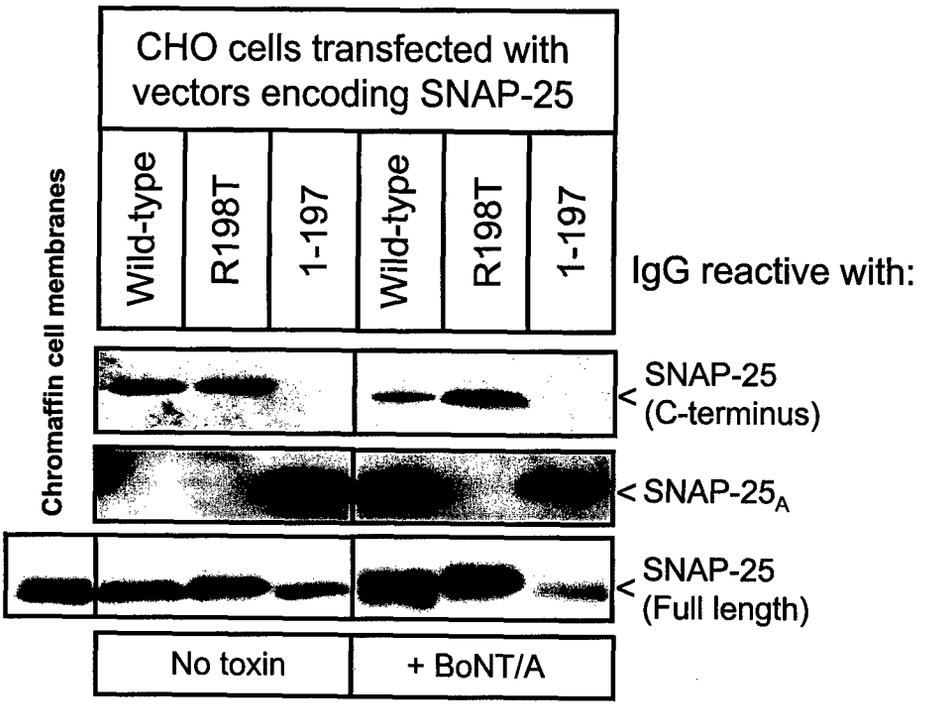

CHO cells transfected with vectors encoding SNAP-25
Wild-type
R198T
1-197
Wild-type
R198T
1-197
IgG reactive with:
Chromaffin cell membranes
SNAP-25 (C-terminus)
SNAP-25A
SNAP-25 (Full length)
No toxin
+ BoNT/A

Figure 9

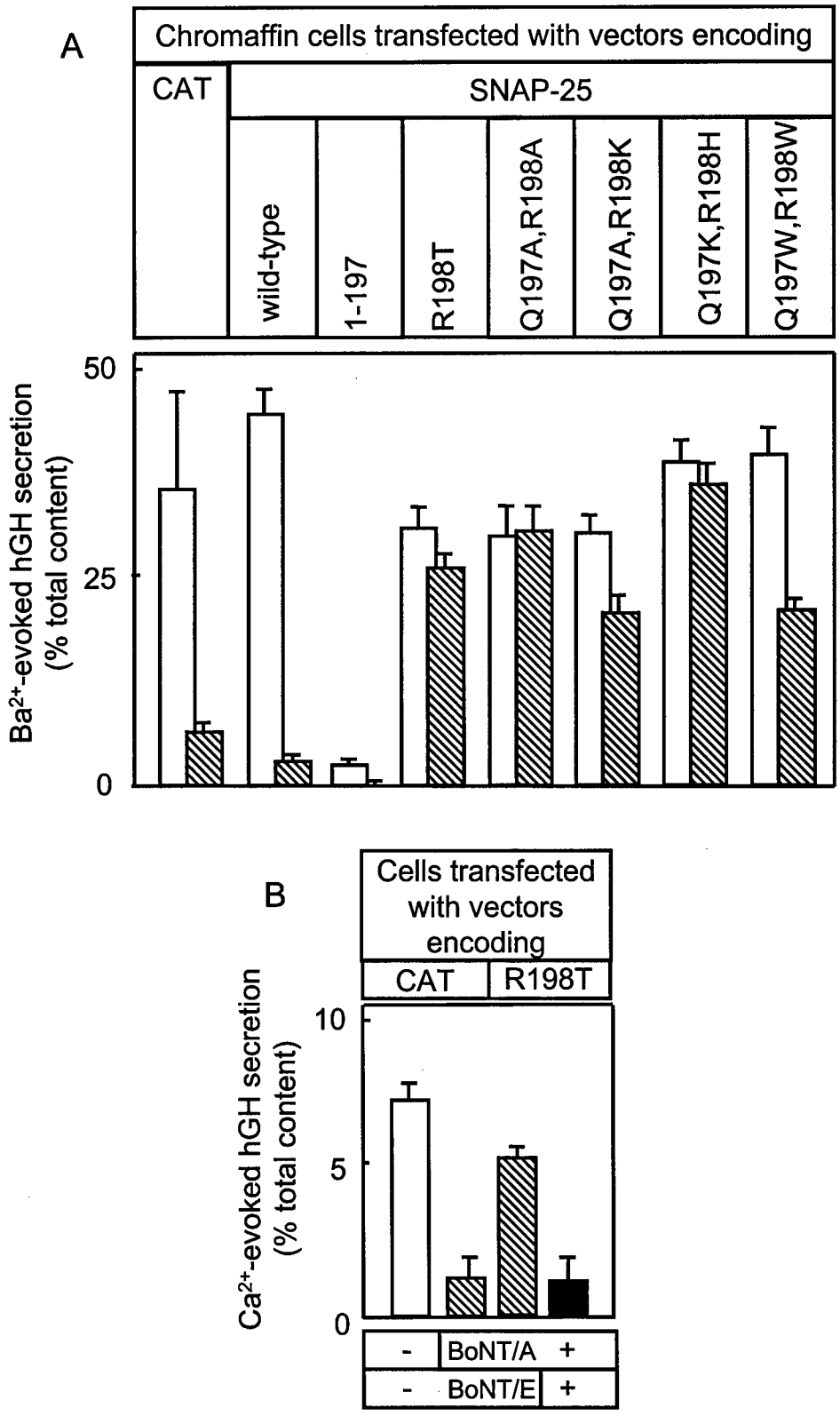
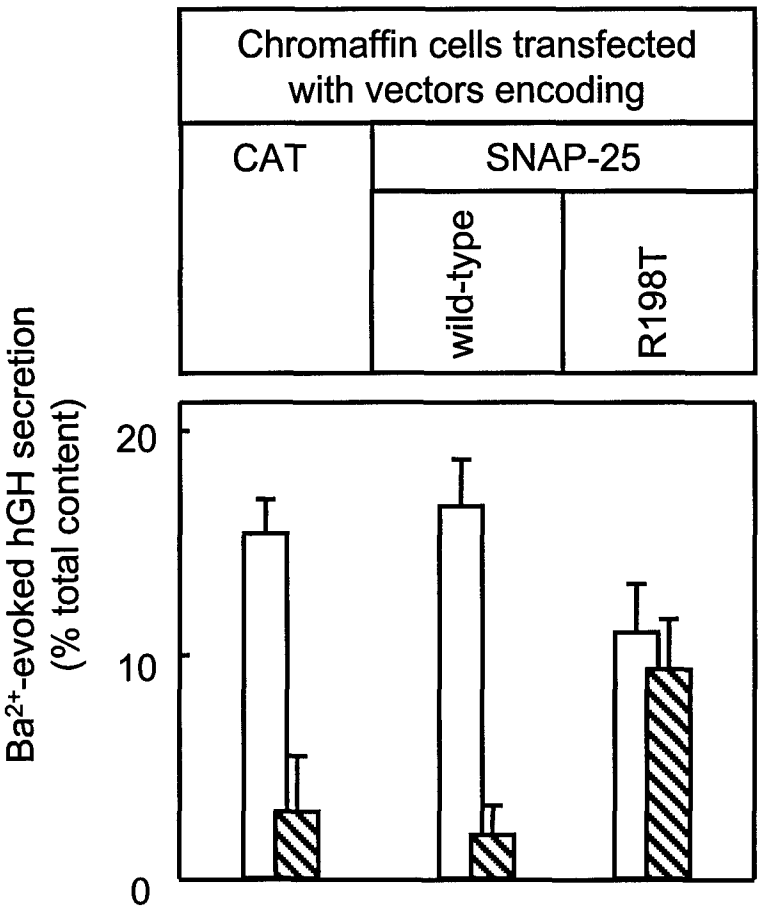

A
Chromaffin cells transfected with vectors encoding
CAT
SNAP-25
wild-type
1-197
R198T
Q197A,R198A
Q197A,R198K
Q197K,R198H
Q197W,R198W
Ba2+-evoked hGH secretion (% total content)
50
25
0
B
Cells transfected with vectors encoding
CAT
R198T
Ca2+-evoked hGH secretion (% total content)
10
5
0
- BoNT/A +
- BoNT/E +

Figure 10


Chromaffin cells transfected with vectors encoding
CAT
SNAP-25
wild-type
R198T
Ba2+-evoked hGH secretion (% total content)
20
10
0

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Protein Kinase B Stimulates the Translocation of GLUT4 but Not GLUT1 or Transferrin Receptors in 3T3-L1 Adipocytes by a Pathway Involving SNAP-23, Synaptobrevin-2, and/or Cellubrevin*

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An interaction of SNAP-23 and syntaxin 4 on the plasma membrane with vesicle-associated synaptobrevin-2 and/or cellubrevin, known as SNAP (soluble *N*-ethyl-maleimide-sensitive factor attachment protein) receptors or SNAREs, has been proposed to provide the targeting and/or fusion apparatus for insulin-stimulated translocation of the GLUT4 isoform of glucose transporter to the plasma membrane. By microinjecting 3T3-L1 adipocytes with the *Clostridium botulinum* toxin B or E, which proteolyzed synaptobrevin-2/cellubrevin and SNAP-23, respectively, we investigated the role of these SNAREs in GLUT4, GLUT1, and transferrin receptor trafficking. As expected, insulin stimulated the translocation of GLUT4, GLUT1, and transferrin receptors to the plasma membrane. By contrast, a constitutively active protein kinase B (PKB-DD) only stimulated a translocation of GLUT4 and not GLUT1 or the transferrin receptor. The GLUT4 response to PKB-DD was abolished by toxins B or E, whereas the insulin-evoked translocation of GLUT4 was inhibited by approximately 65%. These toxins had no significant effect on insulin-stimulated transferrin receptor appearance at the cell surface. Thus, insulin appears to induce GLUT4 translocation via two distinct routes, only one of which involves SNAP-23 and synaptobrevin-2/cellubrevin, and can be mobilized by PKB-DD. The PKB-, SNAP-23-, and synaptobrevin-2/cellubrevin-independent GLUT4 translocation pathway may involve movement through recycling endosomes, together with GLUT1 and transferrin receptors.

In muscle, adipose tissue, and 3T3-L1 adipocytes, insulin primarily increases glucose uptake by promoting the trafficking of vesicles containing GLUT4 (glucose transporter isoform 4) to the plasma membrane (reviewed in Refs. 1 and 2). In the resting state, the majority of GLUT4 resides in vesicles distributed throughout the cell interior, with a fraction of GLUT4 undergoing cycling between the plasma membrane and several intracellular sorting compartments. Insulin triggers a substantial translocation of GLUT4-containing vesicles to the plasma membrane, a phenomenon that can largely explain the increase in V_{max} of the glucose uptake observed. A detailed molecular description of how insulin promotes this translocation is presently lacking, although some of the components involved in the signaling process and the fusion events have been identified.

Binding of insulin to its receptor activates the integral tyrosine kinase, which then elicits a cascade of cellular signaling responses, including phosphorylation of the cytosolic proteins of the insulin-receptor-substrate family (reviewed in Ref. 3). As a consequence of tyrosine phosphorylation, insulin-receptor substrates-1 and -2 bind several effectors; the resultant activation of phosphatidylinositide (4,5)-bisphosphate kinase (PI3-kinase)[1] is of particular importance because it is known to play a key role in transducing the insulin signal leading to GLUT4 vesicle translocation (4–6). The lipid product of this enzyme, namely phosphatidylinositide 3,4,5-trisphosphate, promotes the phosphorylation and activation of the serine/threonine-kinase called protein kinase B (PKB; also known as RAC or Akt) *via* two protein kinases named PDK1 and PDK2 (7). Overexpression of constitutively active forms of PKB causes increased glucose uptake and GLUT4 translocation in adipocytes in the absence of insulin (8, 9), suggesting that this protein kinase may also be a crucial mediator of the effect of insulin on glucose transport, at least in part.

The molecular mechanism of GLUT4 vesicle fusion with the plasma membrane appears to share considerable similarity with Ca^{2+}-evoked exocytosis in neurons and neuroendocrine cells (reviewed in Ref. 10). In neurons, Söllner and co-workers (11) have proposed that the core of the synaptic-clear vesicle docking/fusion complex comprises two plasma membrane proteins, syntaxin 1 and SNAP-25 (synaptosomal protein with a molecular mass of 25 kDa), which interact with synaptobrevin (Sbr) on the vesicle. This provides the targeting specificity and/or the fusion apparatus necessary for neurotransmitter release at the active zones of nerve endings. Additionally, these three proteins serve as receptors for the cytosolic factors *N*-ethyl-maleimide-sensitive factor and SNAP (soluble *N*-ethyl-maleimide-sensitive factor attachment protein), which collectively regulate the ternary associations and, therefore, are referred to as SNAREs (SNAP receptors). SNAP-25 and syntaxin 1, located predominantly on the targeted plasma mem-

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[1] The abbreviations used are: PI3-kinase, phosphatidylinositide (4,5)-bisphosphate kinase; BoNT, botulinum neurotoxin; Cbr, cellubrevin; GFP, green-fluorescent protein; IRAP, insulin-responsive amino peptidase; TfR, transferrin receptor; hTfR, human TfR; HA, hemagglutinin; PKB, protein kinase B; Sbr, synaptobrevin; TeTx, tetanus toxin; TRITC, tetramethylrhodamine β-isothiocyanate; GTPγS, guanosine 5′-3-*O*-(thio)triphosphate; PIPES, 1,4-piperazinediethanesulfonic acid.

brane, are referred to as t-SNAREs, whereas vesicle located Sbr is the v-SNARE (11). An involvement of Sbr-2 and/or its ubiquitous non-neuronal homologue, Cbr (12), and syntaxin 4 (13) in insulin-stimulated GLUT4 vesicle fusion in 3T3-L1 adipocytes has been demonstrated. Their appropriate subcellular locations and protein associations support such a role (14–21); also, the process is inhibited by the cytosolic delivery of selective antibodies against syntaxin 4 (14) and overexpression of interfering SNARE mutants (16, 22). Recently, Martin and co-workers (23) demonstrated that introduction of Sbr-2, but not Sbr-1 or Cbr proteins, into these cells could block insulin-stimulated translocation. Moreover, botulinum neurotoxin (BoNT) serotypes B and D or tetanus toxins (TeTx), which selectively proteolyze Sbr isoforms and Cbr (see below), have been instrumental in establishing the involvement of these v-SNAREs in insulin-induced glucose uptake and GLUT4 trafficking (21, 24–26).

TeTx and serotypes A to G of BoNT are similar but immunologically distinct proteins produced by *Clostridium tetani* and *Clostridium botulinum*, respectively. They are composed of a heavy chain and light chain linked by a disulphide bond and noncovalent interactions. The heavy chain is responsible for high affinity binding to neuronal cholinergic ecto-acceptors, subsequent internalization, and translocation of the active moiety into the cytosol, where the light chain blocks exocytosis (reviewed in Refs. 27–30). The light chains of BoNTs and TeTx are Zn^{2+}-dependent endoproteases (reviewed in Refs. 28–30). Sbr is proteolyzed by TeTx and BoNT/B,/D,/F and/G (reviewed in Refs. 28–30); Cbr is also cleaved by TeTx and BoNT/B (12, 31). BoNT/A and/E proteolyze SNAP-25 at separate C-terminally located sites, whereas BoNT/C1 cleaves both syntaxin 1 (reviewed in Refs. 28–30) and SNAP-25 (32, 33).

Using highly sensitive Western blotting methods, we (21) and others (18, 20, 25, 34, 35) have been unable to detect any SNAP-25 in 3T3-L1 adipocytes. This led to the suggestion that the recently cloned (from human (36) and mouse (37, 38)), non-neuronal homologue termed SNAP-23 (also referred to as Syndet), which is found at high levels and at the appropriate plasma membrane location in 3T3-L1 adipocytes (21, 37, 38), may substitute for SNAP-25 to provide the high affinity ternary docking/fusion complex described (39, 40). The association of SNAP-23 with Sbr-2/Cbr and syntaxin isoforms demonstrated *in vitro* (36, 37, 41) is consistent with a potential role of SNAP-23 in the fusion between GLUT4 vesicles and the plasma membrane. Indeed, recently, Rea and colleagues (42) have demonstrated that insulin-stimulated GLUT4 translocation to the plasma membrane was partially blocked upon the introduction into 3T3-L1 adipocytes of either anti-SNAP-23 antibodies or a synthetic peptide corresponding to the last 24 C-terminal amino acids of SNAP-23.

In this study we have investigated the mechanism by which insulin and a constitutively active PKB (PKB-DD) induce the translocation of GLUT4 to the plasma membrane of 3T3-L1 adipocytes. BoNT/B and BoNT/E, which specifically cleave Sbr-2/Cbr and SNAP-23, respectively, completely blocked the effect of PKB-DD but only partially blocked the effect of insulin. We also found that insulin, but not PKB-DD, caused GLUT1 and TfR translocation, in a manner that was insensitive to the actions of BoNT/B and/E. Collectively, our results support the hypothesis (16, 23, 42, 43) that insulin may operate via two distinct pathways to promote GLUT4 vesicle fusion with the plasma membrane. In addition, our data suggest that only one of these trafficking pathways can be mobilized by a constitutively active PKB.

EXPERIMENTAL PROCEDURES

Materials—A murine 3T3-L1 fibroblast clone (obtained from ATCC; number CCL 92.1) was supplied by the European Collection of Animal Cell Cultures (Salisbury, UK). Tissue culture reagents were from Life Technologies, Inc. or Sigma. High purity digitonin was purchased from Novabiochem (town, UK). Affinity-isolated anti-species-specific Ig conjugated with horseradish peroxidase, insulin and all other reagents were obtained from Sigma. Rabbit anti-GLUT1 antibodies were a gift of Dr. S. Baldwin (University of Leeds). A monoclonal antibody specific for human transferrin receptor (hTfR) was provided by Dr. C. Holmes (University of Bristol), and a plasmid (pCMV5·hTfR) encoding the hTfR cDNA under the control of the cytomegalovirus promoter was kindly provided by Dr. H. Mellor (University of Bristol). The plasmid pGFP-GLUT4 has been described elsewhere (44). The pCMV5 vector was also used to overexpress wild-type and constitutively active (-DD; containing substitutions of Thr^{308} and Ser^{473} for aspartic acid residues) forms of PKB (45), and were kindly provided by Drs. Brian Hemmings (Friedrich Miescher Institute, Basel) and Dario Alessi (University of Dundee).

Antibodies were raised in rabbits against a soluble recombinant His_6-tagged form of mouse syntaxin 1A, lacking the last 27 C-terminal residues, and were affinity-purified on the immobilized antigen (32). Affinity-purified Ig raised against residues 33–94 of human Sbr-2 (a region of amino acid sequence shared with Sbr and Cbr; Ref. 12) was prepared, as detailed elsewhere (32). Because only poor sequence identities exist between Sbr-2/VAMP-2 and the other recently identified mammalian VAMP isoforms 4–8 (46), their immunoreactivity with this antibody is not expected. Moreover, VAMP isoforms 4–8 are unlikely to be proteolyzed by BoNT/B because of their sequence diversites (47). Likewise, a rabbit antiserum to the C terminus of SNAP-25 (residues 195–206) was produced and affinity-purified (48). Antisera were generated in rabbits against peptides corresponding to the C-terminal 11 residues of human SNAP-23 (ANARAKKLIDS); specific Ig was affinity-isolated from immune sera using the immunogenic peptide coupled via its additional N-terminal cysteine to iodoacetyl-Sepharose 4B (prepared as detailed in Ref. 48). Each affinity-purified Ig was tested to ensure that it labeled the requisite protein of appropriate M_r on Western blots in a manner that was preventable by inclusion of the immunogen (data not shown).

Isolated BoNT/A, BoNT/B, BoNT/C1, and BoNT/E were purified and fully nicked, where necessary, as described previously (21, 48, 49); all were shown to be of high purity by sodium dodecyl sulfate-polyacrylamide gel electrophoresis with Coomassie staining of protein and found to exhibit the high levels of toxicity (assessed by mouse bioassay) as published.

Permeabilization of Cells with Digitonin to Observe the Proteolytic Activities of BoNT/A, BoNT/B, BoNT/C1, or BoNT/E—Bovine chromaffin cells were prepared from adrenal glands and maintained in culture, as detailed elsewhere (50). Differentiated 3T3-L1 adipocytes were prepared as outlined before (21). Cells (0.5×10^6 cells/well) were washed three times with buffer A (136 mM NaCl, 4.7 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM $CaCl_2$, 1.2 mM NaH_2PO_4, 5 mM $NaHCO_3$, 10 mM HEPES, pH 7.4). They were then permeabilized at 37 °C using 40 μM digitonin in KGEP buffer (139 mM K^+ glutamate, 20 mM PIPES-HCl, pH 7.0, 1 mM EGTA, 2 mM $MgCl_2$, 2 mM ATP, 0.25 mM phenylmethylsulfonyl fluoride, 20 μg/ml leupeptin, and 10 μg/ml pepstatin A) in the absence or presence of prereduced BoNT/A, BoNT/B, BoNT/C1, or BoNT/E (at the concentrations given in the figure legends). More than 95% of the cells (of the three different lines employed) exhibited nuclear staining by trypan blue, confirming the efficacy of permeabilization. BoNTs were reduced with 20 mM dithiothreitol in 25 mM HEPES, pH 7.4, containing 0.15 M NaCl for a minimum of 30 min at 37 °C before dilution in digitonin-KGEP and application to cells; neurotoxin-free control cells were exposed to the same final concentration of dithiothreitol, which never exceeded 1 mM. After incubation, the medium was aspirated and replaced with 50 mM $NaHCO_3$, pH 8.5, containing protease inhibitors and the cells were harvested and homogenized (detailed in Ref. 31). The homogenate was adjusted to 0.32 M sucrose and centrifuged at $800 \times g$ for 10 min, and the resultant supernatant was subjected to $390{,}000 \times g$ for 1 h to sediment all remaining membranes. The resultant pellets were solubilized in 100 mM Tris, pH 6.8, containing 2% (w/v) SDS plus 1 mM EDTA and solubilized by heating to 90 °C for 10 min.

Immunoblotting and Quantitation of Antigens—Samples were subjected to immunoblotting as detailed in Ref. 21. Antibodies bound to the membranes were detected with anti-species-specific Ig conjugated to horseradish peroxidase and visualized by enhanced chemiluminescence, using the ECL™ detection system (Amersham Pharmacia Bio-

tech). The blots were quantified by densitometric scanning, using image analysis software (National Institutes of Health Image version 1.61), and the resultant values were expressed as a percentage of the toxin-free control. In addition, standard curves of the various amounts of SNARE proteins plotted against band intensity were found to be linear.

Cell Culture, Adipocyte Differentiation, and Microinjection—3T3-L1 fibroblasts were grown on polylysine-coated glass coverslips, and once the adipocyte phenotype was attained (using procedures detailed in Ref. 44), they were equilibrated in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) myoclone plus fetal calf serum, 25 mM HEPES, pH 7.4, and 2 mM $NaHCO_3$, and microinjected using an Eppendorf semi-automatic system. Plasmids were microinjected at 20–100 μg/ml in the absence or presence of prereduced BoNT/B (2 μM) or BoNT/E (1 μM) in 10 mM HEPES, pH 7.5, containing 2 mM $MgCl_2$, 10 mM dithiothreitol, and 50 μM $ZnSO_4$. It is estimated that approximately 10% of the cell volume is delivered during microinjection, thus providing an intracellular concentration of toxin of 100–200 nM. After microinjection, the cells were incubated at 37 °C in Dulbecco's modified Eagle's medium containing 10% (v/v) myoclone plus fetal calf serum for 16–24 h. Before further manipulation, adipocytes were incubated for 2 h at 37 °C in serum-free Dulbecco's modified Eagle's medium and then for 1 h in the presence of 200 nM insulin, as required.

Immunofluorescence Analysis—In some experiments, the cellular distribution of expressed PKB (which possesses a HA tag) was determined. Cells were fixed and permeabilized, using 4% paraformaldehyde and 1% Triton X-100. Unless otherwise stated, all subsequent steps were performed in phosphate-buffered saline supplemented with 3% bovine serum albumin. In some experiments, the cells were stained with monoclonal anti-HA antibodies (10 μg/ml of HA11; Berkeley Antibody Company, CA) for 30 min, followed by incubation in a 1:200 dilution of fluorescein isothiocyanate-conjugated goat anti-mouse IgG (Sigma) or TRITC-conjugated rabbit anti-mouse IgG (Dako Laboratories) for 30 min. In other experiments, PKB was visualized using a 1:200 dilution of a rabbit polyclonal anti-HA antiserum (Santa Cruz) followed by incubation in 1:200 dilution of TRITC-conjugated goat anti-rabbit IgG (Sigma) for 30 min.

To detect the insulin-responsive aminopeptidase, IRAP, the fixed and permeabilized cells were stained with a rabbit polyclonal anti-IRAP serum (5 μg/ml; a kind gift of Drs. Susanna Keller and Gus Lienhard) in phosphate-buffered saline with 3% bovine serum albumin for 30 min. This was followed by a 1:200 dilution of TRITC-conjugated goat anti-rabbit IgG for 15 min. GLUT1 was immunostained using the same procedure but with 25 μg/ml rabbit anti-GLUT1 antibody for 1 h followed by a 1:200 dilution of TRITC-conjugated goat anti-rabbit IgG for 30 min.

Transferrin receptors were detected by staining fixed cells with undiluted monoclonal anti-transferrin receptor hybridoma supernatant in phosphate-buffered saline. Depending on the nature of the experiment, this was followed by a 1:200 dilution of TRITC-conjugated rabbit anti-mouse IgG or fluorescein isothiocyanate-conjugated goat anti-mouse IgG (Sigma) for 30 min in phosphate-buffered saline with 3% bovine serum albumin.

Confocal Microscopy and Image Analysis—Confocal microscopy was performed with a Leica DM IRBE inverted confocal microscope controlled with TCS-NT4 software (Leica). Images were processed with Adobe Photoshop 3.0 and Freelance Graphics 95 (Lotus). The extent of GFP-GLUT4 translocation to the plasma membrane was quantified by marking a region of interest around the exterior and interior faces of the plasma membrane in the confocal image. The levels of fluorescence intensity within these areas were then computed using TCS-NT software, and the intensity of plasma membrane localized GFP-GLUT4 fluorescence (F_{PM}) expressed as a percentage of total cellular GFP-GLUT4 fluorescence intensity (F_T). This method corrects for variations in cell shape, size, and the expression level of GFP-GLUT4.

RESULTS

Murine, but Not Human, SNAP-23 Is Proteolyzed by BoNT/E—To investigate the role of SNAP-23 in GLUT4 vesicle docking and fusion with the adipocyte plasma membrane, the susceptibilities of the murine and human isoforms to proteolytic attack by BoNT/E were examined. A digitonin-based permeabilization method was used to introduce the toxin into cells, because it is known to allow efficient cleavage of other SNAREs (outlined in Ref. 21). Following incubation in the absence or presence of BoNTs, permeabilized cells were lysed and subjected to immunoblotting using primary antibodies reactive

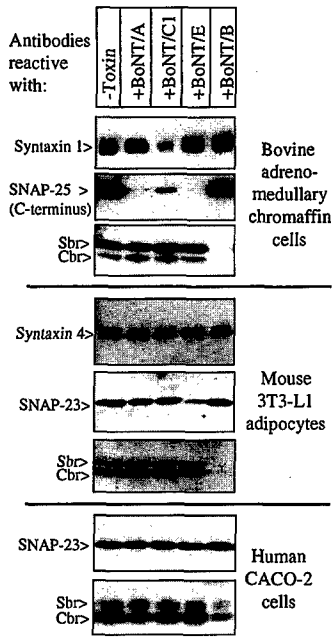

FIG. 1. **Proteolysis of murine SNAP-23 but not the human isoform by BoNT E.** Bovine adrenomedullary chromaffin cells, 3T3-L1 adipocytes, or human CACO-2 cells were incubated with digitonin-KGEP permeabilization buffer at 37 °C for 30 min in the absence or presence of 100 nM of the specified prereduced BoNT serotype. A total membrane fraction was prepared, and equal amounts of protein (20 μg for chromaffin cells and 50 μg for 3T3-L1 or CACO-2 cells) were immunoblotted using the antibodies indicated. Binding of primary antibodies was detected with horseradish peroxidase-conjugated secondary antibodies and visualized using enhanced chemiluminescence (see "Experimental Procedures").

with the relevant SNAREs (detailed in "Experimental Procedures"). Indeed, application of BoNT/A, BoNT/C1, or BoNT/E to permeabilized bovine chromaffin cells resulted in cleavage of the majority of SNAP-25, type C1, additionally, proteolyzed syntaxin 1, whereas BoNT/B proteolyzed the Sbr isoforms and Cbr present (Fig. 1). Also, BoNT/B gave nearly complete cleavage of Cbr and Sbr in permeabilized mouse 3T3-L1 adipocytes and human CACO-2 cells (Fig. 1). These data confirm the high proteolytic activities and appropriate substrate selectivities of the BoNT serotypes used in this study.

The cleavage of SNAP-23 at its C terminus by BoNT/E was investigated using an antiserum that recognizes the C-terminal 11 amino acids downstream of the putative cleavage site in murine SNAP-23 (potentially between Lys^{185}-Ile^{186} (21)); the expectation was that this cleavage would result in the disappearance of its immunoreactivity on Western blots. Whereas treatment of permeabilized mouse and human cells for 30 min with 100 nM of either BoNT/A or BoNT/C1 failed to produce significant cleavage of either murine or human SNAP-23, an identical exposure to BoNT/E diminished the level of reactivity of murine SNAP-23 (Fig. 1). Quantitation by densitometric scanning revealed that BoNT/E removed 76.8 ± 2.9% (mean ± S.D from four separate experiments) of the immunoreactive SNAP-23 present, compared with toxin-free control. In contrast to this extensive cleavage of murine SNAP-23, BoNT/E consistently failed to yield detectable proteolysis of SNAP-23 in the human CACO-2 cell line, under the same conditions (Fig. 1); however, a very slow rate of cleavage cannot be excluded. Furthermore, BoNT/E did not alter the abundance of Sbr-2/Cbr or syntaxin isoforms 1 or 4 in any of these cell lines, reaffirming its known selective proteolytic action (Fig. 1). The observed insensitivity of syntaxin 4 to proteolytic attack by type C1 (Fig. 1) confirms earlier studies (51).

A Requirement for Zn^{2+} and Prereduction of the Interchain

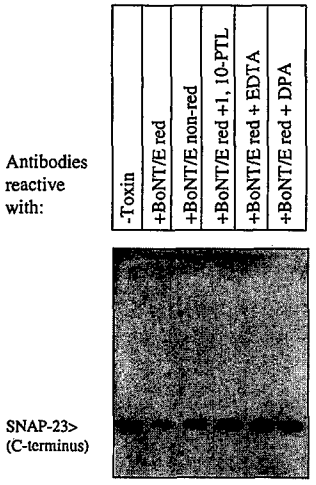

FIG. 2. **Proteolysis of mouse SNAP-23 by BoNT/E requires Zn^{2+} and reduction of the interchain disulphide bond of the toxin.** 3T3-L1 adipocytes were permeabilized in digitonin-KGEP buffer at 37 °C for 30 min in the absence or presence of 100 nM of either prereduced (*red*) or nonreduced (*non-red*) BoNT/E. The reduced toxin was preincubated with the chelator specified (1,10-phenanthroline (*PTL*), 2 mM; EDTA, 2.5 mM; or dipicolinic acid (*DPA*), 2 mM) for 30 min at 37 °C in digitonin-KGEP buffer (lacking 2 mM $MgCl_2$) prior to application to the cells. Equal amounts of membrane protein were immunoblotted using an antiserum specific for the C terminus of SNAP-23 as described under "Experimental Procedures."

Disulphide of BoNT/E for Proteolysis of Murine SNAP-23 Establishes Its Selective Action—To reliably confirm that murine SNAP-23 is cleaved by BoNT/E, we investigated two physical requirements for the proteolytic activities of the other BoNT serotypes shared by type E, namely that Zn^{2+} is essential and that reduction of the interchain disulphide bridge is necessary to activate the protease of the light chain (reviewed in Refs. 28–30). As shown in Fig. 2, prereduction of BoNT/E was found to be a prerequisite for proteolysis of SNAP-23 in permeabilized 3T3-L1 adipocytes. Moreover, incubation of reduced BoNT/E with the divalent cation chelators EDTA, dipicolinic acid, and 1,10-phenanthroline (which is highly selective for Zn^{2+}, compared with Ca^{2+} or Mg^{2+}) before introduction into 3T3-L1 adipocytes removed the ability of BoNT/E to cleave SNAP-23 (Fig. 2); in contrast, the activity of the toxin was not altered by the protease inhibitors included in the permeabilization buffer to inhibit a broad range of other cellular proteolytic enzymes (see "Experimental Procedures"). Therefore, the unique protease activity of BoNT/E must be responsible for cleavage of murine SNAP-23 in 3T3-L1 adipocytes.

Murine SNAP-23 Is Not as Efficiently Proteolyzed as SNAP-25 by BoNT/E—Digitonin-permeabilized chromaffin cells that express SNAP-25 and 3T3-L1 adipocytes that contain SNAP-23 were exposed to various concentrations of BoNT/E. The extents of proteolysis of the respective targets were determined essentially as described in the legend to Fig. 1, by densitometic scanning of the resultant Western blots. The concentrations of BoNT/E extrapolated from the plot in Fig. 3 (*circles*) required to cleave 50% of SNAP-25 was 0.3 nM; in contrast, this extent of cleavage of murine SNAP-23 in permeabilized adipocytes required 30 nM BoNT/E (Fig. 3, *squares*). Thus, BoNT/E is 100-fold less effective in cleaving murine SNAP-23 than its homologue, SNAP-25. Nevertheless, BoNT/E could diminish the SNAP-23 content by 90.3 ± 2.8% (mean ± S.D., $n = 3$). While 2.4 nM BoNT/C1 was sufficient to proteolyze 50% of SNAP-25, longer term treatment of permeabilized adipocytes with high concentrations of this toxin (300 nM) caused insignificant proteolysis of murine SNAP-23 (Fig. 3). A small reduction (approximately 20%) in murine SNAP-23 by BoNT/A

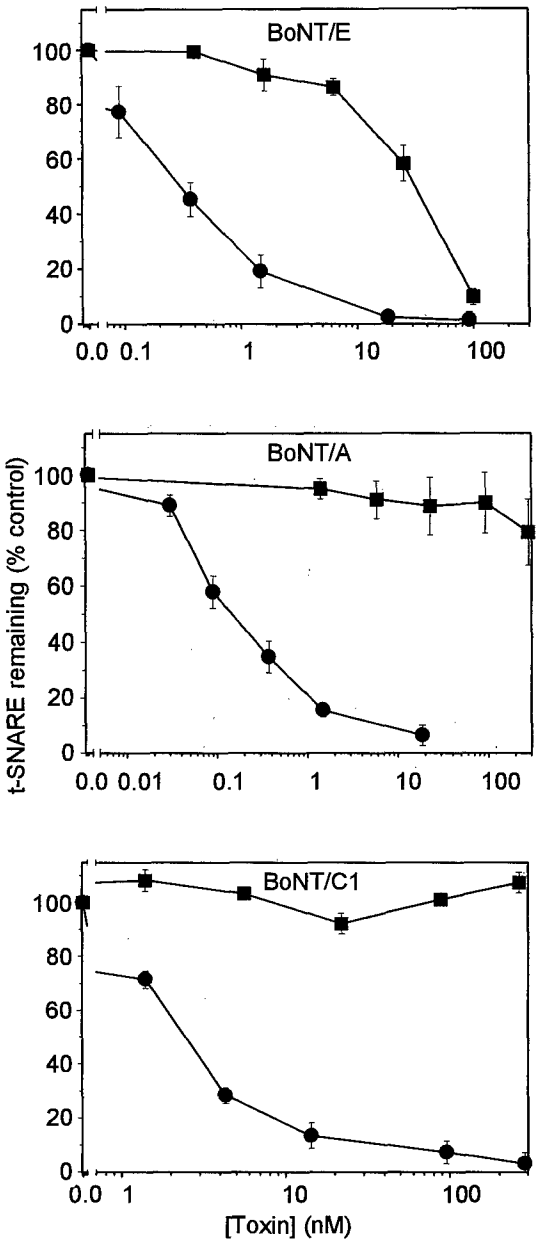

FIG. 3. **Relative susceptibilities of SNAP-25 and murine SNAP-23 to BoNTs.** Prereduced BoNT/E, BoNT/A, or BoNT/C were incubated in digitonin-KGEP buffer for 45 min at 37 °C with either bovine chromaffin cells (source of SNAP-25; *circles*) or murine 3T3-L1 fibroblasts (containing SNAP-23; *squares*). After terminating the proteolytic activity of the toxins using divalent cation chelators, postnuclear membrane fractions were harvested. Equal amounts of membrane protein were subjected to immunoblotting using the appropriate antibodies, and the relative amounts of primary antibody bound were visualized using methods specified in the legend to Fig. 1. The amount of substrates remaining after BoNT treatments were quantified by densitometry and expressed as means (± S.D.; n = 3–5) relative to toxin-free control incubations.

(280 nM) was observed (Fig. 3); however, because 0.04 nM of BoNT/A gave an equivalent degree of proteolysis of SNAP-25, cleavage of SNAP-23 requires approximately 7000 times more toxin. Thus, the ability of BoNT/E to selectively proteolyze SNAP-23, as well as the incubation conditions necessary to attain its efficient cleavage, have been established.

To conclude, our observations confirm the reported proteolysis of bacterially expressed murine SNAP-23 by native BoNT/E (52), although another group found murine recombinant SNAP-23 to be resistant to the toxin (25). The inability of

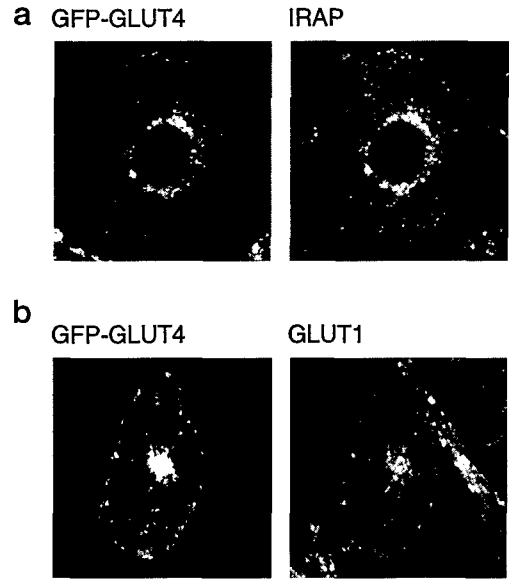

FIG. 4. **GFP-tagged GLUT4, IRAP, and GLUT1 co-localization in 3T3-L1 adipocytes.** *a*, 3T3-L1 adipocytes were microinjected with plasmid encoding GFP-GLUT4 and imaged 24 h later after fixing and staining with an anti-IRAP antibody, which was detected using TRITC-conjugated anti-rabbit IgG. *b*, adipocytes were microinjected with the plasmid encoding GFP-GLUT4 and imaged 24 h later after fixing and staining with an anti-GLUT1 antibody that was detected using TRITC-conjugated anti-rabbit IgG. The figure shows representative laser scanning confocal micrographs of the distribution of these proteins.

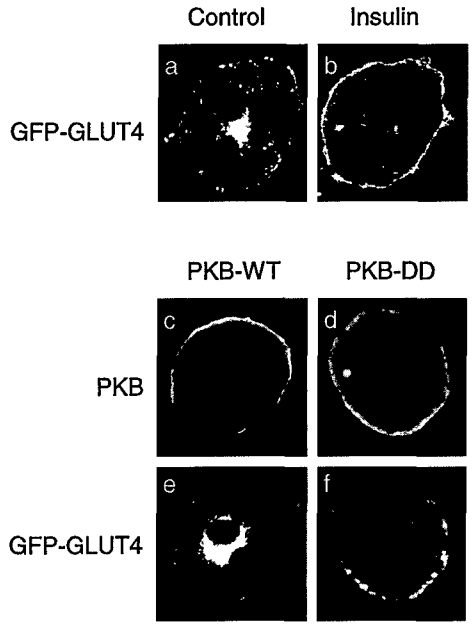

FIG. 5. **Insulin and constitutively active PKB promote GFP-GLUT4 translocation.** 3T3-L1 adipocytes were microinjected with a plasmid encoding GFP-GLUT4 and imaged 24 h later after incubation in the absence (*a* and *c–f*) or presence (*b*) of 200 nM insulin for 1 h. Alternatively, the cells were additionally co-microinjected with plasmids directing the overexpression of wild-type PKB (*c* and *e*) or constitutively active PKB-DD (*d* and *f*). These cells were fixed and, in the case of *c* and *d*, stained with anti-HA antibodies that recognize the HA-tag on the expressed PKBs. Visualization of GFP-GLUT4 fluorescence (*a*, *b*, *e*, and *f*) or anti-HA immunofluorescence using TRITC (*c* and *d*) were performed by laser scanning confocal microscopy. Selected representative cells are shown.

this toxin to proteolyze human SNAP-23 agrees with the finding of others (53). SNAP-23, while being 59% identical in amino acid sequence to SNAP-25, exhibits extensive diversity at its C terminus (36–38), the region which encompasses the cleavage sites of BoNT/A, BoNT/C1, and BoNT/E. Thus only small changes in amino acid sequence between SNAP-25 and human SNAP-23 could account for the inabilities of BoNT/A, BoNT/C1, or BoNT/E to proteolyze the latter. Similarly, human and murine forms of SNAP-23 also differ by 13% (36–38), perhaps explaining their differing proteolytic susceptibilities to BoNTs.

GFP-GLUT4 Trafficking to the Plasma Membrane Occurs in Response to a Constitutively Active Form of PKB—Having demonstrated the cleavage of SNAP-23 by BoNT/E, its effect on GLUT4 translocation was investigated after microinjection into 3T3-L1 adipocytes. To monitor GLUT4 translocation, we used a single cell assay that involved expression of a chimera linking GLUT4 to the C terminus of GFP from the jellyfish, *Aequoria victoria* (44). This entailed microinjecting a plasmid encoding GFP-GLUT4 into the nucleus, followed by laser scanning confocal microscopy 24 h later. We confirmed that the GFP-GLUT4 chimera was expressed in native GLUT4-containing vesicles, by demonstrating its efficient co-localization with endogenous IRAP, a *bona fide* GLUT4 vesicle-resident protein (54, 55). As shown in Fig. 4*a*, the majority of the GFP-GLUT4-containing vesicles co-localized with IRAP. In addition, GFP-GLUT4 was found in GLUT1-containing vesicles (presumably endosomes) but also in a population of vesicles that largely lacked GLUT1 (Fig. 4*b*). This is consistent with the known apparent distribution of GLUT4 between endosomes and specialized GLUT4-containing vesicles as determined previously (56, 57). In the basal state, the bulk of the GFP-tagged GLUT4 was distributed throughout the cytoplasm and just beneath the plasma membrane but was also concentrated close to the nucleus (Fig. 5*a*). Exposure to insulin resulted in a dramatic redistribution of GFP-GLUT4, such that a continuous line of fluorescence was observed on the plasma membrane (Fig. 5*b*; see also Ref. 44).

Adipocytes were also co-microinjected with pGFP-GLUT4 and plasmids encoding either wild-type PKB or a mutant PKB rendered constitutively active through substitution of Thr^{308} and Ser^{473} for aspartate residues (PKB-DD; both PKB constructs possessed a HA epitope tag for subsequent detection). The cells were fixed, stained with anti-HA antibodies, and imaged 24 h post-microinjection. Interestingly, both wild-type PKB and PKB-DD were located largely on the plasma membrane (Fig. 5, *c* and *d*); this is different from the predominantly cytosolic distribution of this kinase in nonstimulated fibroblasts and adipocytes as determined by either immunofluorescence staining or cell extraction followed by subcellular fractionation (9, 58–60). The reasons for the discrepancy are not known but could reflect the ability of the pleckstrin homology domain of PKB to bind phosphatidylinositide (4,5)-bisphosphate (61), which may be more abundant in the plasma membrane of resting 3T3-L1 adipocytes. Nevertheless, a plasma membrane location of the wild-type protein *per se* is not sufficient to promote the translocation of GLUT4-containing vesicles (Fig. 5*e*). PKB-DD, but not wild-type PKB, caused GFP-GLUT4 translocation to the plasma membrane to a similar extent to that induced by insulin (*i.e.* compare Fig. 5, *f versus b*). Thus, PKB activation mimicked by substitution of Thr^{308} and Ser^{473} for aspartate residues can promote GLUT4 translocation.

BoNT/B and /E Block GLUT4 Translocation Induced by Constitutively Active PKB More Efficiently than That Evoked by Insulin—Because 100 nM BoNT/E was required to cause approximately 90% proteolysis of SNAP-23 in permeabilized 3T3-L1 adipocytes in a 45-min incubation (Fig. 3), concentrations of between 100 and 200 nM were delivered by microinjection. Because BoNTs can exert their activities over long periods

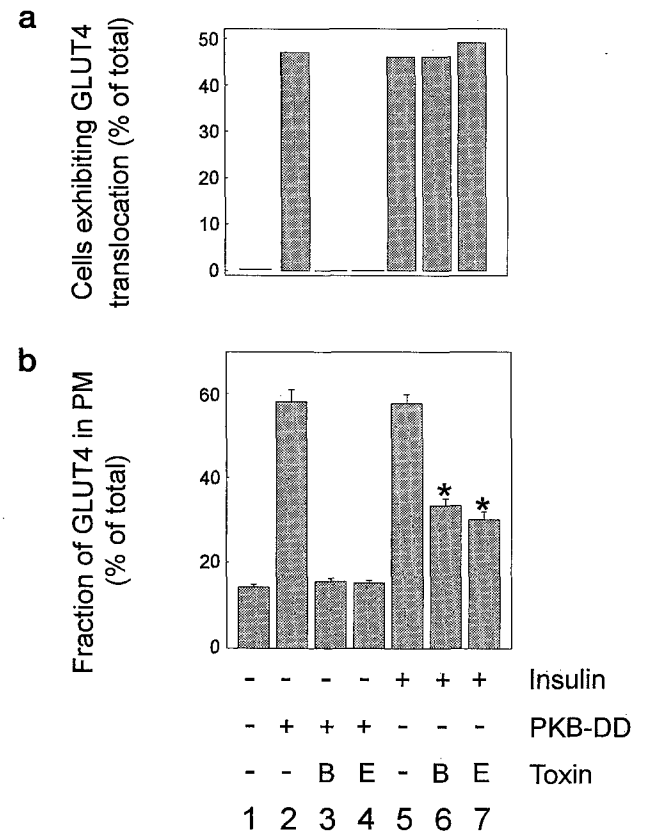

FIG. 6. **BoNT/B and BoNT/E block GFP-GLUT4 translocation in 3T3-L1 adipocytes.** 3T3-L1 adipocytes were microinjected with pGFP-GLUT4 and where appropriate PKB-DD, in the presence of prereduced BoNT/B (*B*) or BoNT/E (*E*) or toxin-free buffer (−) as indicated. The cells were serum starved for 2 h and treated, as indicated, in the absence or presence of 200 nM insulin for 1 h. The cells were fixed, permeabilized, and stained with anti-HA antibodies to confirm the expression and location of HA-tagged PKB-DD. The cells were then examined both visually and by laser scanning confocal microscopy. In *a*, cells that were deemed visually to have undergone a translocation response (*i.e.* exhibited a continuous line of GFP fluorescence around the plasma membrane) are expressed as the percentage of all cells examined. In *b* the data (means ± S.E.) is plotted as the fraction of total GFP-GLUT4 fluorescence found in the plasma membrane, calculated from a confocal image of the cell (see "Experimental Procedures"). Because fewer than 50% of the cells responded to PKB-DD or insulin (*a*), in *b* only those cells that exhibited a translocation response were used in the quantitation in conditions 2, 5, 6, and 7. In both panels, the results are pooled from two independent experiments, and each bar comprises data from 20–44 cells. * indicates p values of <0.001 *versus* toxin-free insulin-stimulated controls.

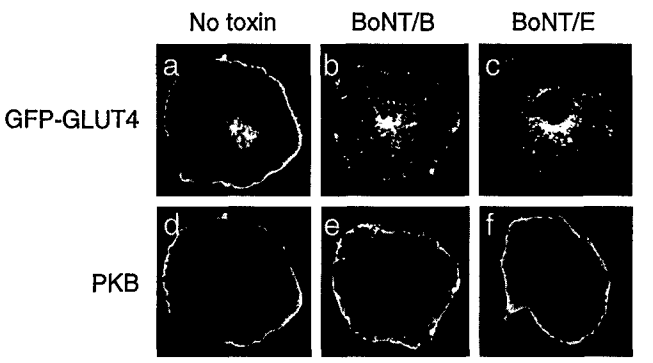

FIG. 7. **BoNT/B and BoNT/E block GFP-GLUT4 translocation in response to constitutively active PKB.** 3T3-L1 adipocytes were microinjected with plasmids encoding GFP-GLUT4 and constitutively active PKB-DD (*a–f*), in the absence (*a* and *d*) or presence of prereduced BoNT/B (*b* and *e*) or BoNT/E (*c* and *f*). After 24 h, the cells were fixed and immunostained with anti-HA antibodies to visualize the expressed PKB-DD. The figure shows fluorescence micrographs obtained by confocal microscopy. GFP-GLUT4 distribution is visualized in *a–c*. *d–f* illustrate the distribution of PKB detected using HA-staining.

in mammalian cells (reviewed in Refs. 21 and 30),[2] most of the SNAP-23 would be cleaved by type E during the following 24 h period, while the GFP-GLUT4 was expressed from the co-injected GFP-GLUT4 plasmid. In validation of this approach, a previous study found that insulin-stimulated glucose-uptake in BoNT/B-treated 3T3-L1 adipocytes remains maximally inhibited for at least 48 h (21). Subsequently, the effect of the toxin on GFP-GLUT4 translocation induced by PKB-DD or insulin was examined. In parallel experiments, the involvement of Sbr-2/Cbr was investigated after administration of BoNT/B at a concentration known in these cells to cause near complete proteolysis of Sbr-2 and Cbr. At the end of the experiments, the cells were fixed and stained with anti-HA antibody to confirm, where necessary, the expression and subcellular location of PKB variants.

The translocation of GFP-tagged GLUT4 was quantified in two ways. First, we visually examined each cell and determined whether they exhibited a continuous ring of fluorescence around the plasma membrane, indicative of translocation. On this basis, in the basal state, none of the cells showed a "translocated phenotype" (Fig. 6*a*). It was found that insulin and PKB-DD induced GFP-GLUT4 translocation in a similar fraction of the cells (Fig. 6*a*). (The reason underlying the heterogeneity in the insulin response is not known but has been noted in our previous studies on both insulin-stimulated GLUT4 translocation (44) and gene transcription (62), as well as by all others using the plasma membrane lawn technique to assess GLUT4 translocation.) As a second measure of the extent of GFP-GLUT4 translocation, the amount of GFP-GLUT4 found at the plasma membrane was calculated as a fraction of the total cellular GFP-GLUT4 measured. Thus, insulin and PKB-DD also caused a similar increase (approximately 4-fold) in the level of plasma membrane GFP-GLUT4.

The ability of PKB-DD to promote GLUT4 translocation to the plasma membrane in toxin-free cells (Figs. 6*b* and 7*a*) was completely blocked in the presence of microinjected BoNT/B (Figs. 6*b* and 7*b*) or BoNT/E (Figs. 6*b* and 7*c*), whereas the expressed PKB-DD was again mostly confined to the plasma membrane (Fig. 7, *d–f*). In the case of insulin, although neither toxin appeared to reduce the proportion of cells that responded to insulin (Fig. 6*a*), the fraction of GFP-GLUT4 that translocated in each individual cell was reduced by approximately 65% by either toxin (Fig. 6*b*). This partial inhibition of the insulin-stimulated GLUT4 translocation event by BoNT/B is consistent, therefore, with our previous studies (21). Similarly, Rea *et al.* (42) reported that a C-terminal peptide from SNAP-23 or specific antibodies reactive to the latter only blocked insulin-stimulated GLUT4 translocation by approximately 40% in permeabilized cells (see "Discussion").

Insulin, but Not PKB-DD, Stimulates the Translocation of the Transferrin Receptor and GLUT1—The data presented above suggest that insulin stimulates GLUT4 translocation *via* two distinct pathways, only one of which is blocked by BoNT/B or BoNT/E. The latter may represent the trafficking pathway that responds to the introduction of the constitutively active PKB-DD mutant. We thus investigated the nature of the toxin-insensitive GLUT4 trafficking pathway.

In the basal state, GLUT4 is found in a vesicle compartment termed a GLUT4 storage vesicle (43), which contains Sbr-2 but is apparently devoid of GLUT1 and the TfR, as well as in the recycling endosomal pool that also contains the TfR and

[2] P. G. P. Foran, L. M. Fletcher, P. B. Oatey, N. Mohammed, J. O. Dolly, and J. M. Tavaré, unpublished observations.

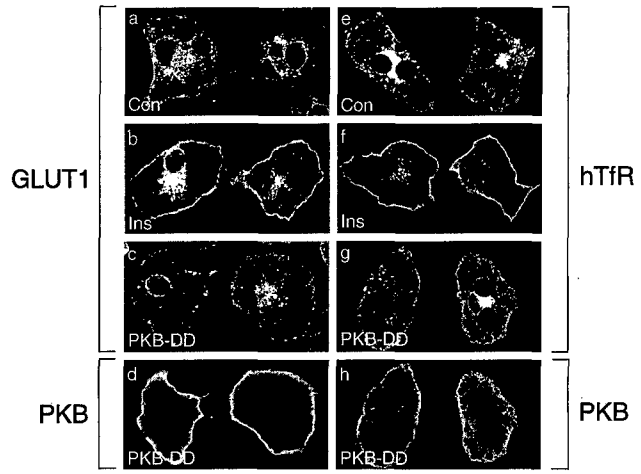

FIG. 8. **GLUT1 and transferrin receptor translocation are stimulated by insulin but not by PKB-DD.** 3T3-L1 adipocytes were microinjected in the absence or presence of plasmids encoding the human transferrin receptor (*e–h*) and PKB-DD (*c, d, g,* and *h*). 24 h later the cells were incubated in the absence (*a, c, d, e, g,* and *h*) or presence (*b* and *f*) of 200 nM insulin for 1 h prior to fixation and staining with polyclonal anti-GLUT1 antibodies (*a–c*) or murine monoclonal anti-hTfR antibodies (*e–g*). The cells in *c* and *g* were co-stained with rabbit polyclonal antibodies reactive with the HA epitope present on the expressed PKB to give *d* and *h,* respectively. Visualization of GLUT1 and transferrin receptors (with TRITC) or PKB-DD (using fluorescein isothiocyanate) was performed by laser scanning confocal microscopy. Selected representative cells are shown.

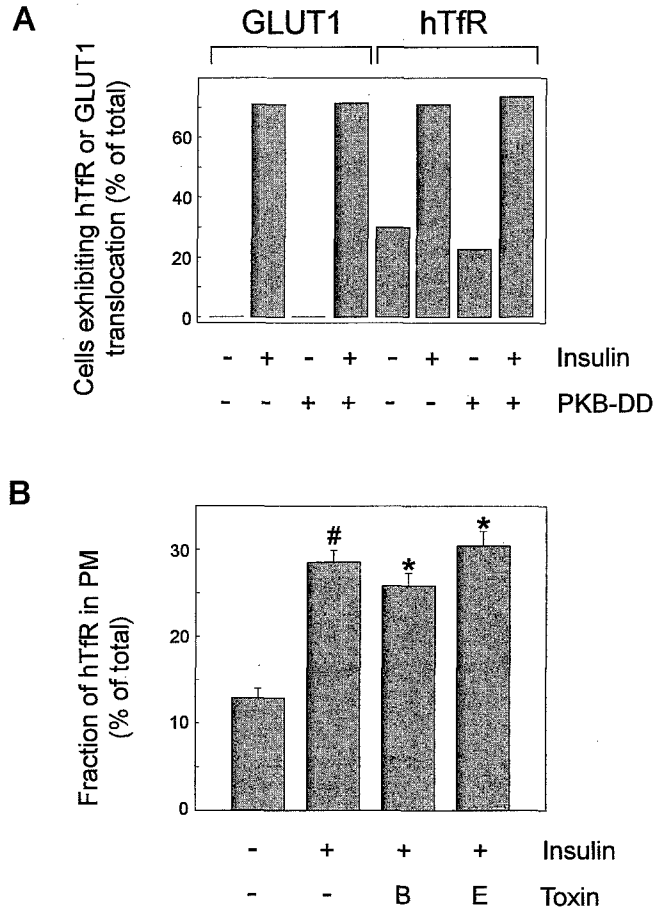

FIG. 9. **Insulin, but not PKB-DD, stimulates GLUT1 and transferrin receptor translocation to the plasma membrane of 3T3-L1 adipocytes, responses that are resistant to the action of BoNTs.** In *a,* 3T3-L1 adipocytes were microinjected with or without pCMV5·hTfR where appropriate and in the absence or presence of PKB-DD as indicated. After 24 h the cells were serum starved for 2 h and treated in the absence or presence of 200 nM insulin for 1 h, as specified. The cells were fixed, permeabilized and, where specified, stained with anti-GLUT1 or anti-hTfR antibodies. The cells were then examined both visually and by laser scanning confocal microscopy. Those that exhibited a continuous line of GLUT1- or transferrin receptor-derived fluorescence around the plasma membrane were deemed to have undergone translocation. These data are expressed as the percentages of cells examined that underwent translocation. Each bar comprises at least 65 cells pooled from at least two separate experiments. In *b,* 3T3-L1 adipocytes were microinjected with pCMV5·hTfR and, where indicated, with prereduced BoNT/B or BoNT/E or toxin-free buffer (−). The cells were treated in the absence or presence of insulin and were then fixed, permeabilized, and stained with anti-hTfR antibodies. All the cells expressing hTfR were then examined by laser scanning confocal microscopy, and the amount of hTfR located in the plasma membrane was calculated as a fraction of the total cellular content. The data are expressed as means ± S.E. Each *bar* represents data from at least 150 cells, pooled from at least two separate experiments. # indicates p values <0.001 *versus* toxin-free basal controls. * indicates that no significant statistical difference exists relative to insulin-treated toxin-free controls.

GLUT1 (Refs. 23, 56, and 57; see also "Discussion"). Therefore, we investigated the effect of insulin or PKB-DD on GLUT1 and TfR translocation. Immunofluorescence staining showed that the majority of cells exhibited relatively low levels of endogenous GLUT1 on the plasma membrane in the basal state, *i.e.* few cells exhibited a *continuous* ring of GLUT1 immunofluorescence in the plasma membrane (Figs. 8*a* and 9*a*). Insulin treatment caused an increase in GLUT1 translocation such that approximately 70% of the cells exhibited a continuous ring of GLUT1 immunoreactivity at the plasma membrane (Figs. 8*b* and 9*a*). Conversely, expression of the constitutively active PKB-DD had no apparent effect on the subcellular distribution of GLUT1 or the ability of insulin to cause a translocation to the plasma membrane (Figs. 8, *c* and *d*, and 9*a*). The extent of GLUT1 translocation was quantified and expressed as the fraction of cellular GLUT1 present in the plasma membrane (% of total). In cells expressing wild-type PKB, 8.9 ± 0.9% (n = 46) of the GLUT1 was expressed in the plasma membrane (mean ± S.E. for the number of cells shown in parentheses and pooled from three independent experiments). In the presence of PKB-DD but the absence of insulin, 6.2 ± 0.6% (n = 41) of the GLUT1 was found in the plasma membrane; however, this increased to 22.1 ± 1.7% (n = 37) in the simultaneous presence of insulin and PKB-DD (p < 0.001 *versus* control without insulin).

To investigate the distribution of the TfR and because our antisera do not detect endogenous murine TfR, 3T3-L1 adipocytes were microinjected with a plasmid encoding the hTfR. This was subsequently detected by immunofluorescence staining with a highly avid monoclonal antibody specific for the human form of the receptor. In the basal state, the expressed hTfR was predominantly located intracellularly (Fig. 8*e*) and exhibited a considerable co-localization with endogenous GLUT4 (data not shown), although this was incomplete, as would be expected (57). Unlike GLUT1, however, in some cells (approximately 20–30%; Fig. 9*b*) a continuous ring of hTfR immunofluorescence was found in the plasma membrane probably as a result of overexpression. Exposure to insulin caused a pronounced translocation of hTfR to the plasma membrane such that approximately 70% of the cells now exhibited a continuous ring of hTfR around the plasma membrane (Figs. 8*f* and 9*a*). But, as with GLUT1, the constitutively active PKB-DD mutant did not promote any detectable translocation of hTfR to the plasma membrane or alter the ability of insulin to cause translocation of the latter (Figs. 8*g* and 9*a*).

The effects of BoNT/B and BoNT/E on insulin-stimulated transferrin receptor translocation were quantified; the ectopic expression of the hTfR allowed us to easily detect the toxin injected cells. However, neither BoNT had any significant ef-

fect on insulin-stimulated translocation of hTfR (Fig. 9*b*). In support of the validity of our results, a recent study by Martin and co-workers (23) found that transferrin-horseradish peroxidase-mediated ablation of recycling TfR-containing endosomes inhibited the subsequent insulin-stimulated translocations of TfR, as well as GLUT1. Despite this, insulin was still able to elicit large increases in the cell surface levels of GLUT4, presumably through activation of an alternate vesicle trafficking pathway (*i.e.* distinct from the fusion of recycling endosomes with the plasma membrane).

DISCUSSION

The molecular basis underlying the ability of insulin to promote the translocation of GLUT4 and thus stimulate glucose uptake remains incompletely defined. Our results comparing the effects of insulin and a constitutively active PKB mutant, together with the use of two botulinum toxins, support the proposal (16, 23, 42, 43, 57) that at least two pathways are involved in insulin-stimulated GLUT4 trafficking to the plasma membrane. Notably, our results suggest that only one of these pathways is sensitive to the action of a constitutively active PKB.

Insulin stimulated the translocation of GLUT1, GLUT4, and the TfR to the plasma membrane of 3T3-L1 adipocytes. This contrasted with the effect of overexpressing a constitutively active PKB-DD mutant (rendered active *via* substitution of the PDK1 and PDK2 phosphorylation sites with aspartate residues), which promoted only a translocation of GLUT4 but not GLUT1 or TfR. The effect of PKB-DD on GLUT4 translocation is consistent with the known effects on glucose transport of the constitutively active gag-Akt oncogene and a PKB mutant possessing a myristoylation signal sequence at its N terminus (8, 9). Although this suggests that PKB activation can mimic insulin-stimulated GLUT4 translocation, its precise role in the effect of insulin remains controversial. For example, Cong and co-workers (63) found that a dominant-negative PKB blocked insulin-stimulated glucose transport by approximately 20%, whereas Kitamura *et al.* (64) found no apparent effect. Regardless of this controversy, constitutively active PKB-DD is clearly a useful tool to investigate the mechanism by which insulin may promote GLUT4 translocation.

We investigated the role of SNARE proteins in the insulin- and PKB-mediated translocations of GLUT4, GLUT1, or TfR. Numerous studies have highlighted the essential requirement of syntaxin 4 and Sbr-2/Cbr for insulin-stimulated GLUT4 vesicle translocation in adipocytes (see the Introduction). To form a high affinity interaction between vesicle-derived Sbr and plasma membrane syntaxin-1, SNAP-25 is required (39, 40). However, numerous laboratories (20, 25, 34, 35, 38), including our own (see the Introduction) have been unable to detect the expression of SNAP-25 in 3T3-L1 adipocytes, despite the use of extremely sensitive Western blotting and high affinity selective antibodies. This prompted us to investigate the role of SNAP-23, which exhibits the appropriate molecular characteristics (see the Introduction), is abundant in 3T3-L1 adipocytes and was reported by James and colleagues (42) to be involved in insulin-stimulated glucose uptake.

BoNT/E cleaves murine, but not human, SNAP-23 in permeabilized cells, albeit at 100-fold higher concentrations than required for equivalent cleavage of SNAP-25. However, when employed at a sufficient concentration, BoNT/E can proteolyze >90% of SNAP-23, thus enabling an assessment of the role of this SNARE in GLUT4 vesicle trafficking. To do this, we microinjected BoNT/B or BoNT/E and performed single cell imaging of GLUT4 translocation using a GFP-tagged GLUT4 construct. Although insulin-stimulated GLUT4 translocation to the plasma membrane was partially blocked (approximately 65%) by BoNT/E, intriguingly this toxin completely blocked the effect of PKB-DD on GLUT4 translocation (Figs. 6 and 7). The same result was obtained following introduction of BoNT/B into cells to cleave the Sbr-2/Cbr present. Collectively, these observations suggest that PKB-dependent GFP-GLUT4 translocation is completely dependent on Sbr-2/Cbr and SNAP-23.

Although a variety of methods have been used to interfere with SNARE interactions in adipocytes, only partial inhibitions (maximally between 65–80%) of insulin-stimulated glucose uptake and GLUT4 translocation have been reported. These include the use of botulinum or tetanus toxins (21, 24, 26), interfering SNARE fragments, and peptides (14, 16, 22, 42) or inhibitory anti-SNARE antibodies (14, 42). Failure to achieve a complete blockade of the insulin response thus led to the proposal that insulin uses both BoNT-sensitive and -insensitive pathways to promote GLUT4 translocation. Our data suggest that PKB-DD exclusively stimulates GLUT4 translocation via the BoNT-sensitive pathway and from a vesicle pool that lacks GLUT1 and TfR. Such a vesicle population probably represents the post-endocytic GLUT4 storage compartment termed GLUT4 storage vesicles by Rea and James (43) and that resembles small synaptic vesicles with respect to its SNARE content and recycling characteristics.

Although GLUT4 and GLUT1 have been shown to segregate into distinct vesicle pools in adipocytes (56, 56, 66), there is also considerable evidence to suggest that a substantial fraction of GLUT4 (approximately 40–50% (56, 57)) also resides in recycling endosomes that contain GLUT1 and the TfR. Insulin-stimulated translocation of GLUT1 from this latter pool to the plasma membrane occurs independently of Sbr-2/Cbr and SNAP-23 (16, 23, 42), observations that are consistent with our own data in which neither BoNT/B nor BoNT/E blocked insulin-stimulated TfR translocation (Fig. 9*b*). It is curious to note that despite the known presence of Cbr in approximately 60% of transferrin receptor-containing vesicles in 3T3-L1 adipocytes (57), we observe no significant inhibition of insulin-stimulated translocation when Cbr function is prevented. Although an earlier study (21) suggested that Cbr may mediate insulin-stimulated GLUT4 translocation, more recently Martin and co-workers (23) found that only Sbr-2 was likely to be involved. Therefore, the precise function of Cbr in 3T3-L1 adipocytes is yet to be defined.

Although the exocytosis of GLUT1- and TfR-containing vesicles recycling via the endocytic pathway is not sensitive to the actions of PKB-DD (Fig. 8), inhibitors of PI3-kinase have been reported to block insulin-stimulated translocation of the transferrin receptor (67) and GLUT1 (4). Taken together with our own data, this suggests that PI3-kinase is central to the translocation of GLUT4 from both pools. However, the signal may then diverge such that PKB then mobilizes GLUT4 to the plasma membrane from the GLUT4 storage vesicles and an as yet unknown pathway stimulates mobilization of GLUT4, GLUT1, and the TfR from recycling endosomes. If the two pools of GLUT4 are in dynamic equilibrium, in the presence of dominant-negative PKB, GLUT4 may be able to traffic to the plasma membrane by returning into recycling endosomes. The extent to which insulin utilizes these two trafficking mechanisms in any one cell type and the degree to which these pools might equilibrate may help to explain why dominant-negative PKBs only partially suppress insulin-stimulated GLUT4 translocation (63), if they have any effect at all (64). This possibility requires further investigation.

Concluding Comments—Our data lend support to the proposal that GLUT4 translocation occurs via at least two distinct trafficking pathways. One is Sbr-2/Cbr and SNAP-23-dependent and may represent GLUT4 storage vesicles that can be

mobilized to the plasma membrane in response to constitutively active PKB-DD. This pathway may correspond to the pool of GLUT4 in adipocytes reported to resemble small synaptic vesicles in neurons (based on morphology and recycling characteristics). The other trafficking pathway is Sbr-2/Cbr- and SNAP-23-independent (or can at least use alternative BoNT-insensitive SNARE proteins) and is not stimulated by PKB-DD. The latter pathway may represent the translocation of GLUT4 present in GLUT1- or TfR-containing endosomes.

Other trafficking pathways and/or signaling mechanisms may also exist. For example, GLUT4 translocation induced by osmotic shock or GTPγS in adipocytes or contraction in muscle have been reported to occur independently of PI3-kinase and protein kinase B (22, 65, 68, 69). Clearly, identifying the events between PI3-kinase activation and SNARE-dependent docking/fusion of GLUT4 vesicles, as well as the other "putative" pathways utilized by insulin that may operate independently of Sbr-2/Cbr and SNAP-23, are important avenues for future exploration.

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REFERENCES

1. Holman, G. D., and Kasuga, M. (1997) *Diabetologia* **40,** 991–1003
2. Kandror, K. V., and Pilch, P. F. (1996) *Am. J. Physiol. Endocrinol. Metab.* **34,** E1–E14
3. Yenush, L., and White, M. F. (1997) *Bioessays* **19,** 491–500
4. Clarke, J. F., Young, P. W., Yonezawa, K., Kasuga, M., and Holman, G. D. (1994) *Biochem. J.* **300,** 631–635
5. Tanti, J. F., Gremeaux, T., Grillo, S., Calleja, V., Klippel, A., Williams, L. T., Van Obberghen, E., and Le Marchand-Brustel, Y. (1996) *J. Biol. Chem.* **271,** 25227–25232
6. Martin, S. S., Haruta, T., Morris, A. J., Klippel, A., Williams, L. T., and Olefsky, J. M. (1996) *J. Biol. Chem.* **271,** 17605–17608
7. Alessi, D. R., and Cohen, P. (1998) *Curr. Opin. Genet. Dev.* **8,** 55–62
8. Kohn, A. D., Summers, S. A., Birnbaum, M. J., and Roth, R. A. (1996) *J. Biol. Chem.* **271,** 31372–31378
9. Tanti, F., Grillo, S., Gremeaux, T., Coffer, P. J., Van Obberghen, E., and Le Marchand-Brustel, Y. (1997) *Endocrinology* **138,** 2005–2010
10. Söllner, T. (1995) *FEBS Lett.* **369,** 80–83
11. Söllner, T., Whiteheart, S. W., Brunner, M., Erdjument-Bromage, H., Geromanos, S., Tempst, P., and Rothman, J. (1993) *Nature* **362,** 318–324
12. McMahon, H. T., Ushkaryov, Y. A., Edelmann, L., Link, E., Binz, T., Niemann, H., Jahn, R., and Sudhof, T. C. (1993) *Nature* **364,** 346–349
13. Bennett, M. K., Garcia-Arraras, J. E., Elferink, L. A., Peterson, K., Fleming, A. M., Hazuka, C. D., and Scheller, R. H. (1993) *Cell* **74,** 863–873
14. Volchuk, A., Wang, Q. H., Ewart, H. S., Liu, Z., He, L. J., Bennett, M. K., and Klip, A. (1996) *Mol. Biol. Cell* **7,** 1075–1082
15. Cain, C. C., Trimble, W. S., and Lienhard, G. E. (1992) *J. Biol. Chem.* **267,** 11681–11684
16. Olson, A. L., Knight, J. B., and Pessin, J. E. (1997) *Mol. Cell. Biol.* **17,** 2425–2435
17. Volchuk, A., Sargeant, R., Sumitani, S., Liu, Z., He, L., and Klip, A. (1995) *J. Biol. Chem.* **270,** 8233–8240
18. Jagadish, M. N., Fernandez, O. S., Hewish, D. R., Macaulay, S. L., Gough, K. H., Grusovin, J., Verkuylen, A., Cosgrove, L., Alafaci, A., Frenkel, M. J., and Ward, C. J. (1996) *Biochem. J.* **317,** 945–954
19. Jagadish, M. N., Tellam, J. D., Macaulay, S. L., Gough, K. H., James, D. E., and Ward, C. W. (1997) *Biochem. J.* **321,** 151–156
20. Timmers, K. I., Clark, A. E., Omatsu-Kanbe, M., Whiteheart, S. W., Bennett, M. K., Holman, G. D., and Cushman, S. W. (1996) *Biochem. J.* **320,** 429–436
21. Chen, F., Foran, P., Shone, C. C., Foster, K. A., and Dolly, J. O. (1997) *Biochemistry* **36,** 5719–5728
22. Chen, D., Elmendorf, J. S., Olson, A. L., Li, X., Earp, S., and Pessin, J. E. (1997) *J. Biol. Chem.* **272,** 27401–27410
23. Martin, L. B., Shewan, A., Millar, C. A., Gould, G. W., and James, D. E. (1998) *J. Biol. Chem.* **273,** 1444–1452
24. Cheatham, B., Volchuk, A., Kahn, C. R., Wang, L., Rhodes, C. J., and Klip, A. (1996) *Proc. Natl Acad. Sci. U. S. A.* **93,** 15169–15173
25. Macaulay, S. L., Rea, S., Gough, K. H., Ward, C. W., and James, D. E. (1997) *Biochem. Biophys Res. Commun.* **237,** 388–393
26. Tamori, Y., Hashiramoto, M., Araki, S., Kamata, Y., Takahashi, M., Kozaki, S., and Kasuga, M. (1996) *Biochem. Biophys. Res. Commun.* **220,** 740–745
27. Simpson, L. L. (1986) *Infect. Immun.* **52,** 858–862
28. Montecucco, C., and Schiavo, G. (1993) *Trends Biochem. Sci.* **18,** 324–327
29. Niemann, H., Blasi, J., and Jahn, R. (1994) *Trends Cell Biol.* **4,** 179–185
30. Dolly, J. O. (1992) *in Handbook of Experimental Pharmacology: Selective Neurotoxicity* (Herken, H., and Hucho, F., eds) Vol. 102, pp. 681–717, Springer-Verlag, Berlin
31. Foran, P., Lawrence, G., and Dolly, J. O. (1995) *Biochemistry* **34,** 5494–5503
32. Foran, P., Lawrence, G. W., Shone, C. C., Foster, K., and Dolly, J. O. (1996) *Biochemistry* **35,** 2630–2636
33. Williamson, L. C., Halpern, J. L., Montecucco, C., Brown, J. E., and Neale, E. A. (1996) *J. Biol. Chem.* **271,** 7694–7699
34. Wong, P. P. C., Daneman, N., Volchuk, A., Lassam, N., Wilson, M. C., Klip, A., and Trimble, W. S. (1997) *Biochem. Biophys. Res. Commun.* **230,** 64–68
35. Macaulay, S. L., Hewish, D. R., Gough, K. H., Stoichevska, V., MacPherson, S. F., Jagadish, M., and Ward, C. W. (1997) *Biochem. J.* **324,** 217–224
36. Ravichandran, V., Chawla, A., and Roche P. A. (1996) *J. Biol. Chem.* **271,** 13300–13303
37. Araki, S., Tamori, Y., Kawanishi, M., Shinoda, H., Masugi, J., Mori, H., Niki, T., Okazawa, H., Kubbota, T., and Kasuga, M. (1997) *Biochem. Biophys. Res. Commun.* **234,** 257–262
38. Wang, G., Witkin, J. W., Hao, G., Bankaitis, V. A., Scherer, P. E., and Baldini, G. (1997) *J. Cell Sci.* **110,** 505–513
39. Pevsner, J., Hsu, S.-C., Braun, J. E. A., Calakos, N., Ting, A. E., Bennett, M. K., and Scheller, R. H. (1994) *Neuron* **13,** 353–361
40. Hayashi, T., McMahon, H., Yamasaki, S., Binz, T., Hata, Y., Südhof, T. C., and Niemann, H. (1994) *EMBO J.* **13,** 5051–5061
41. Tellam, J. T., Macaulay, S. L., McIntosh, S., Hewish, D. R., Ward, C. W., and James, D. E. (1997) *J. Biol. Chem.* **272,** 6179–6186
42. Rea, S., Martin, L. B., McIntosh, S., Macaulay, S. L., Ramsdale, T., Baldini, G., and James, D. E. (1998) *J. Biol. Chem.* **273,** 18784–18792
43. Rea, S., and James, D. E. (1997) *Diabetes* **46,** 1667–1677
44. Oatey, P. B., Van Weering, D. H. J., Dobson, S. P., Gould, G. W., and Tavaré, J. M. (1997) *Biochem. J.* **327,** 637–642
45. Alessi, D. R., Andjelkovic, M., Caudwell, B., Cron, P., Morrice, N., Cohen, P., and Hemmings, B. A. (1996) *EMBO J.* **15,** 6541–6551
46. Advani, R. J., Bae, H.-R., Bock, J. B., Chao, Y.-C. D., Prekeris, R., Yoo, J.-S., and Scheller, R. H. (1998) *J. Biol. Chem.* **273,** 10317–10324
47. Shone, C. C., and Roberts, A. K. (1994) *Eur. J. Biochem.* **225,** 263–270
48. Lawrence, G. W., Foran, P., and Dolly, J. O. (1996) *Eur. J. Biochem.* **236,** 877–886
49. Lawrence, G. W., Foran, P., Mohammed, N., DasGupta, B. R., and Dolly, J. O. (1997) *Biochemistry* **36,** 3061–3067
50. Lawrence, G. W., Weller, U., and Dolly, J. O. (1994) *Eur. J. Biochem.* **222,** 325–330
51. Schiavo, G., Shone, C. C., Bennett, M. K., Scheller, R. H., and Montecucco, C. (1995) *J. Biol. Chem.* **270,** 10566–10570
52. Washbourne, P., Pellizzari, R., Baldini, G., Wilson, M. C., and Montecucco, C. (1997) *FEBS Lett.* **418,** 1–5
53. Sadoul, K., Berger, A., Niemann, H., Weller, U., Roche, P. A., Klip, A., Trimble, W. S., Regazzi, R., Catsicas, S., and Halban, P. A. (1997) *J. Biol. Chem.* **272,** 33023–33027
54. Kandror, K. V., and Pilch, P. F. (1994) *Proc. Natl Acad. Sci. U. S. A.* **91,** 8017–8021
55. Mastick, C. C., Aebersold, R., and Lienhard, G. E. (1994) *J. Biol. Chem.* **269,** 6089–6092
56. Robinson, L. J., and James, D. E. (1992) *Am. J. Physiol.* **263,** E383—E393
57. Martin, S., Tellam, J., Livingstone, C., Slot, J. W., Gould, G. W., and James, D. E. (1996) *J. Cell Biol.* **134,** 625–635
58. Andjelkovic, M., Alessi, D. R., Meier, R., Fernandez, A., Lamb, N. J., Frech, M., Cron, P., Cohen, P., Lucocq, J. M., and Hemmings, B. A. (1997) *J. Biol. Chem.* **272,** 31515–31524
59. Wijkander, J., Holst, L. S., Rahn, T., Resjo, S., Castan, I., Manganiello, V., Belfrage, P., and Degerman, E. (1997) *J. Biol. Chem.* **272,** 21520–21526
60. Calera, M. R., Martinez, C., Liu, H. Z., El Jack, A. K., Birnbaum, M. J., and Pilch, P. F. (1998) *J. Biol. Chem.* **273,** 7201–7204
61. James, S. R., Downes, C. P., Gigg, R., Grove, S. J., Holmes, A. B., and Alessi, D. R. (1996) *Biochem. J.* **315,** 709–713
62. Rutter, G. A., White, M. R., Tavaré, and J. M. (1995) *Curr. Biol.* **5,** 890–899
63. Cong, L. N., Chen, H., Li, Y., Zhou, L., McGibbon, M. A., Taylor, S. I., and Quon, M. J. (1997) *Mol. Endocrinol.* **11,** 1881–1890
64. Kitamura, T., Ogawa, W., Sakaue, H., Hino, Y., Kuroda, S., Takata, M., Matsumoto, M., Maeda, T., Konishi, H., Kikkawa, U., and Kasuga, M. (1998) *Mol. Cell. Biol.* **18,** 3708–3717
65. Elmendorf, J. S., Chen, D., and Pessin, J. E. (1998) *J. Biol. Chem.* **273,** 13289–13296
66. Zorzano, A., Wilkinson, W., Kotliar, N., Thoidis, G., Wadzinkski, B. E., Ruoho, A. E., and Pilch, P. F. (1989) *J. Biol. Chem.* **264,** 12358–12363
67. Shepherd, P. R., Soos, M. A., and Siddle, K. (1995) *Biochem. Biophys. Res. Commun.* **211,** 535–539
68. Brozinick, J. T., Jr., and Birnbaum, M. J. (1998) *J. Biol. Chem.* **273,** 14679–14682
69. Lund, S., Pryor, P. R., Ostergaard, S., Schmitz, O., Pedersen, O., and Holman, G. D. (1998) *FEBS Lett.* **425,** 472–474